Fall 2007

Nest guarding and aggression in the red-backed salamander, Plethodon cinereus

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NEST GUARDING AND AGGRESSION IN THE RED-BACKED SALAMANDER, *PLETHODON CINEREUS*

BY

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B.S., Ramapo College of New Jersey, 2004

THESIS

Submitted to the University of New Hampshire
In Partial Fulfillment of
the Requirements for the Degree of

Master of Science

in

Zoology

September, 2007

This thesis has been examined and approved.

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August 15, 2007
Date

ACKNOWLEDGEMENTS

Foremost, I would like to thank my advisor, Dr. Michelle P. Scott, for accepting me into the program, for giving me the opportunity to do this research, and for teaching me how to be a scientist. Thanks for all of your patience and for believing in me. I would like to thank Dr. David Berlinsky for allowing me to use his laboratory and for his endless perseverance while we effectuated some of these techniques. Dr. Kimberly Babbitt provided expert knowledge of my study species and helped immensely with the experimental design.

I would also like to extend thanks to J. Specker, L. Houck, D. Forester, R. Jaeger, and E. Crespi for their invaluable input on this project. Additional thanks to Paul Tsang and Stacia Sower for with help with laboratory protocols. Erica Westerman and Michelle Walsh helped edit; and Olivia Tornick helped compile the thesis and photographs. Thanks must also be extended to Diane Lavalliere and Nancy Wallingford for help with administrative and financial questions.

To my friends and family, who are too numerous to list, I could not have done this without your unending love and support. Kudos to my dog Rex, who kept me company in the field in all sorts of weather and cheered me up after many a long day in the lab. Thanks mom, for tolerating a little girl who always insisted on bringing home various creatures in my pockets, much to your chagrin. It was you who instilled in me a wonder and respect for all living things, and imparted to me the confidence to accomplish anything I desire. This work is dedicated to my father, who did not quite survive to see it finished, you will always be in my heart.

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Forward

Reproduction requires a great deal of energy, and individuals are limited in the amount of time and resources they can devote to producing and raising their young. The depletion of energy for any given reproductive event may be detrimental to a parent's physical condition, survival, and future reproductive output. Parental care adds further costs to reproduction which can include an increased risk of injury or death when defending offspring, the loss of mating or feeding opportunities while rearing offspring, an increase in the time to the next reproduction, and fewer future offspring (Trivers, 1972). On the other hand, such expenditure is typically beneficial to the offspring, enhancing their condition, growth, survival, and reproductive success. Parents must weigh the benefits of investing in current reproductive efforts against the costs to future reproduction, and parents need to balance offspring demands against their own self-maintenance (Williams, 1966; Trivers, 1972). Natural selection favors individuals that are able to maximize the difference between the benefits and the costs; therefore parental care is likely to evolve when the benefits are higher than the costs.

The strategy of caring for young is prevalent in the animal kingdom. Parental care is found in a broad range of taxonomic groups, including both invertebrates (i.e. insects and crustaceans) and vertebrates. Care exists in many forms and extends to many stages of the offspring's life. Pre-natal care can include

embryonic nourishment, incubation, nest preparation, egg guarding, and transportation. Post-natal care may incorporate food provisioning, protection of offspring, and teaching of survival skills. Parental care varies by species, and may be provided by the female only, the male only, or by both parents.

Perhaps one of the costliest aspects of parental care (but also one imparting great benefits to offspring) is aggressive defense of offspring against predators and cannibalistic conspecifics. Parents often risk serious injury and even death when guarding against intruders. Because of the great risk, parents should have mechanisms for responding to the relative reproductive value of current offspring and for altering their defensive behavior proportionately. But how do parents know whether to defend or abandon offspring? What parameters do they use to gauge the reproductive value of a brood? Are parents able to evaluate the number of offspring, the developmental stage of eggs, or the time of season and adjust the level of care accordingly? Are they able to assess which intruders pose the greatest threat? How does aggression during nest guarding differ from territorial aggression? Do individual parents behave consistently, for example are some always aggressive and others always passive? Are some individuals more effective at guarding offspring than others? What roles do the age and experience of parents play in affecting their ability to defend offspring? Does a threat to offspring cause physiological changes in the adult in preparation for a fight; or conversely, do individuals with increased hormone titers defend offspring more effectively?

To answer some of these questions about nest guarding, researchers have historically focused on birds and mammals, due to the prevalence of parental behavior in these classes. Other taxa such as fish, reptiles, and amphibians have been examined less frequently. However, since the early 1970's there has been a growing interest in examining animal behavior from evolutionary and comparative perspectives. Therefore, researchers are increasingly looking toward understudied groups to examine parental care. One of the least studied groups in this area has been the class Amphibia, since many amphibians do not exhibit parental care behavior. However, a large percentage of species in the family Plethodontidae (Order Caudata) expend considerable time and energy caring for young, and aggressively defend their eggs pre-hatching. I chose to examine aggression during nest guarding in the red-backed salamander, *Plethodon cinereus,* since females of this species are well known for territorial aggression and brood eggs for a lengthy period, yet little is known about aggression during nest guarding.

In the laboratory, different aspects of aggression during nest guarding were examined in brooding *P. cinereus* females. Several external factors which may affect maternal decisions about nest guarding were examined: clutch size, clutch age, sex of the intruder, and physical size of the attendant. An endocrine correlate of this behavior was also examined by challenging brooding females and analyzing tissue for steroid hormone levels, to determine whether internal (hormonal) factors may also affect aggressive nest guarding behavior.

ABSTRACT

NEST GUARDING AND AGGRESSION IN THE
RED-BACKED SALAMANDER, PLETHODON CINEREUS

By

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University of New Hampshire, September, 2007

Several environmental factors and endocrine mechanisms were examined which may affect the intensity of aggression displayed by female red-backed salamanders (*Plethodon cinereus*) during nest guarding. In a set of behavioral experiments, female were challenged by conspecific intruders during egg guarding and intensity of aggression was scored. Four variables were examined: 1) *clutch age*, 2) *clutch size*, 3) *sex of intruder*, and 4) *body size* of the attendant. In a second set of experiments, tissue samples were taken from challenged and unchallenged brooding females to compare testosterone (T) titers. A novel non-lethal technique was developed for extraction and assay of T using autotomized tail tissue, and a seasonal profile of T for males and females was determined.

My results suggest that: 1) there are consistent behavioral differences among individuals in females' response to an intruder, 2) females defend late-stage eggs more aggressively than early-stage eggs, 3) females aggressively defend both large and small clutches, 4) females are aggressive toward both male and female intruders and 5) aggression is not correlated with body size. T titers are highest in males in autumn when females are competing for mates, and highest in gravid females in spring when they are searching for nest sites. T is significantly higher

in brooding females that are challenged by a conspecific intruder than in unchallenged females.

CHAPTER I

REPRODUCTIVE VALUE, THREAT ASSESSMENT, AND AGGRESSION DURING NEST GUARDING IN THE RED-BACKED SALAMANDER, *PLETHODON CINEREUS*

Abstract.- Defense of young against intruders is an energetically costly and a potentially risky parental behavior. Female red-backed salamanders, *Plethodon cinereus,* guard their clutch for many weeks and aggressively defend eggs against intruders. I used female *P. cinereus* to examine parental decisions regarding nest defense, and focused on four factors to determine their effect on the level of aggression in guarding females: 1) *clutch age*, 2) *clutch size*, 3) *sex of intruder*, and 4) *body size* of the attendant. Invasions of nests of brooding females by conspecifics were staged in the laboratory, and intensity of aggression was scored. Many parental females were highly aggressive under multiple conditions, and there was a significant effect of individual on aggression score. Guarding females were significantly more aggressive when guarding older clutches (6 weeks post-oviposition) than younger clutches (4 weeks post-oviposition). Females were not significantly more aggressive when guarding large (10 egg) versus small (4 egg) clutches. Females were not significantly more aggressive toward female or male intruders, although there was a trend for increased aggression toward females. There was also no relationship between body size and level of aggression.

Introduction

Parental investment theory describes any parental expenditure which increases an offspring's chance of survival at the cost of the parent's ability to invest in future offspring (Clutton-Brock, 1991; Trivers, 1972). An animal should invest in parental care when expected future benefits, such as high offspring survivorship, are substantial (Williams, 1966). When parental care is costly in terms of reduced future reproductive potential, breeding individuals should be sensitive to changes in the value of current offspring and adjust their care accordingly. Such costs typically include increased risk of predation and injury, as well as decreased mating opportunity, fecundity, and foraging time (Fitzpatrick, 1973). Thus, this strategy is not advantageous unless substantial reproductive benefits can be reaped (Williams, 1966).

Studies on many organisms have demonstrated that clutch attendance by parents increases hatchling survivorship (Highton and Savage, 1961; Brumbaugh, 1998; Crespi and Lessig, 2004); therefore this behavior has the potential to increase lifetime fitness of the nest attendant. Animals should respond to proximate cues regarding costs versus benefits by adjusting the level of parental care (Winkler, 1987). Evaluation of clutch characteristics such as clutch size or age, may enable a parent to predict the reproductive value of offspring and may influence parental care choices.

Aggression during nest guarding of eggs or neonates is a particularly costly reproductive strategy in terms of energy expenditure (Ng and Wilbur, 1995). Parents that proactively protect offspring against intruders risk serious injury

(Bachmann,1984) . Many organisms aggressively guard their progeny against conspecific and/or interspecific intruders, suggesting that for these species the benefits of such behavior outweigh the costs. Thus, nest guarding behavior may be a useful gauge with which to examine investment decisions by parents. Few studies have examined the effects of clutch properties, intruder properties, or size of the attendant on aggression during clutch defense. The studies that have examined these factors have primarily focused on avian models (Martin and Horn, 1993) therefore research on other organisms is needed. Within Amphibia, lengthy parental care is an atypical strategy. Only twenty percent of caudates care for young, and the majority of these are members of the family Plethodontidae (Crump, 1995). I used the red-backed salamander, *Plethodon cinereus*, as a model for studying parental decisions, since females exhibit extended parental care.

Study Species.- Plethodon cinereus is a lungless terrestrial salamander, common to deciduous forests of North America. This fully terrestrial life cycle makes *P. cinereus* an excellent test subject; as individuals are easy to collect, maintain, and observe in a laboratory. Female red-backed salamanders live an average of eight to thirteen years (LeClair, 2006) and become sexually mature at two or three years of age (Sayler, 1966). Growth is indeterminate (Marvin, 2001), and the sexes are monomorphic. Females reproduce biennially, as the energy required to produce and attend a clutch is substantial (Sayler, 1966). Broods typically consist of 3-15 large eggs (Bachmann, 1984), which measure approximately 3 to 4 mm in diameter (personal observation). Females remain

sequestered inside decaying logs with their brood for as long as ten weeks (Bachmann, 1984). During brooding, female red-backed salamanders curl their bodies around their eggs, occasionally vibrating them. Tactile stimulation maintains aeration and uniform distribution of egg contents, and prevents desiccation and fungal infection (Forester, 1979). After eggs hatch, females may remain with the neonates for several additional weeks (Bachmann, 1984). Females also actively defend their nests against predation and cannibalization.

Nest Guarding and Aggression.- Extensive studies of *P. cinereus* have demonstrated territorial aggression by both males and females (Jaeger *et al.*, 1982; Jaeger and Schwarz 1991; Jaeger and Peterson, 2002). Its agonistic repertoire has been categorized into easily recognizable threat behaviors such as Head Up, Body Up, Nudging, and Biting (Jaeger and Schwarz, 1991). These distinct postures facilitate scoring of aggressive behavior. Yet, few studies have examined aggressive behavior during nest guarding as these salamanders secretively brood their eggs deep inside log cavities. In addition, it has been difficult to induce oviposition in the laboratory (Houck and Schwenk, 1984; Jockusch, 1996, Bernardo and Arnold, 1999). Although a great deal is known about territorial aggression in this species, very little is known about the aggressive behavior exhibited specifically during nest guarding.

Purpose.- The objective of this study was to investigate parental investment during nest guarding by manipulating several variables effecting aggressive behavior. I examined clutch properties (*clutch age* and *clutch size*), an intruder characteristic (*sex of intruder*), and size of the brooding female (*body size*).

Older and larger clutches should have higher reproductive value than younger and smaller ones; therefore I predicted that such clutches would elicit increased aggression. I predicted that females would defend more strongly against female than against male intruders, since females compete for mates and are known to cannibalize eggs. I also predicted that long and/or heavy females (those with greater energy reserves) would be more aggressive than small females. To test these predictions, I staged invasions by unfamiliar conspecifics of brooding females in their nest cavities in the laboratory during two consecutive summers, 2005 and 2006.

Materials and Methods

Collection and Husbandry.- Salamanders were collected at College Woods and Foss Farm in Durham, N.H. (latitude: 43.14 N, longitude: 70.93 W), in northern hardwood forests consisting mainly of hemlock, oak, and maple, with an abundance of decaying logs used by red-backed salamanders for cover. I collected both striped and lead-backed (unstriped) color morphs by carefully hand searching in fallen logs and under rocks. In spring of 2005, I collected mature male and female (gravid and non-gravid) red-backed salamanders, and females with clutches for use in the *clutch size*, *sex of intruder*, and *body size* experiments. In spring of 2006, I also collected non-gravid females and females with their clutches for use in the *clutch age* experiment. I determined sex and reproductive status of each individual by "candling" since their gonads and eggs are easily visible when backlit (Gillette and Peterson, 2001). Inter-nares distance, the distance between nares at the tip of the snout, was used to further verify sex (Quinn and Graves, 1999). In all experiments only individuals with intact tails were used. Handling of salamanders was minimized to avoid damaging their integument.

All salamanders were housed in covered plastic containers measuring 19 cm x 13 cm x 9.5 cm. Approximately two centimeters of compressed soil covered the bottom of each container. At one end of the container, an inverted 9 cm petri dish covered with a circular piece of brown paper towel served as a den (Brumbaugh, 1998). The towel helped to retain moisture and to keep the den dark. This technique also allowed observation by occasionally lifting the towel

without disturbing the nest site. The containers were kept at 20°C, on a 14L: 10D light cycle, and were misted occasionally to maintain a high relative humidity (Bachmann, 1984). Salamanders were fed white worms (Enchytrae*)* and wingless fruit flies (*Drosophila melanogaster*) several times per week.

Gravid females were acclimated and given time to oviposit eggs in their laboratory housing. Oviposition success was low in 2005, only 4 of 23 gravid females oviposited. This is consistent with other studies which suggest that Plethodontids are often reluctant to lay eggs in the laboratory (Houck and Schwenk, 1984; Jockusch, 1996, Bernardo and Arnold, 1999). To obtain enough clutches and attendants in 2005, 24 additional females with their eggs were collected from the field in June and July. I photographed oviposited eggs under a dissecting microscope every 3-4 days to document the developmental stages (Appendix B). In 2006, I compared field collected clutches to developmental photos from 2005 to estimate the date of oviposition for the *clutch age* experiment.

A few of the females ate or abandoned one or more of their eggs (in 2005, N=4; in 2006, N=3); and a small number of clutches failed due to fungal infection (in 2005, N=2; in 2006, N=0). Data were retained in the analysis if females continued to guard partial clutches. In nests where clutches were viable, the females stayed sequestered with their eggs and gently moved them occasionally. Most brooding females continued to feed opportunistically.

At the conclusion of the experiments, all animals and their offspring were released to their original collection sites in September of 2005 and 2006.

Behavioral Observation.- Based on previously documented aggressive postures and behaviors (Jaeger and Schwarz, 1991; Mathis *et al.*, 2000; Davis 2002; Forester, 1983) as well as careful preliminary laboratory observations, an ethogram was developed for scoring the level of aggression displayed by brooding females during staged nest site invasions (Table 1.1). Aggressive behaviors increase in intensity from one (guard eggs) through nine (bite hold). The rationale for this scoring system is based on the increasing effectiveness of each behavior in deterring an intruder; for example bites are more effective than nudges (Forester, 1983). Level of aggression was quantified by the sum of all acts times the score for each act. This sum was used in analyses as the aggression score for each individual. The number of snaps (score of 7) and bites (score of 8 or 9) (the most aggressive behaviors) were summed and analyzed separately. To account for the behavior of the intruder, I calculated an adjusted aggression score by dividing the aggression score by the number of times the intruder approached the resident. In addition, to account for possible misinterpretation of the hierarchical nature of this scoring system and to confirm my results, I also divided resident's behavior into one of two categories for statistical analysis: low aggression (no behavior with a score above 4), or high aggression (any behavior with a score of 5 or above). Latency (time in seconds) to first aggressive act (any behavior scoring 5 or above) was analyzed using t-tests assuming unequal variance or paired t-tests.

Behavioral observations took place between the hours of 8:00 p.m. and midnight, and were conducted under red light. Container covers and petri dishes

Score	Behavior	Description
1	Guard Eggs	Moving to or curling around clutch
2	Head Up	Lifting head above neutral
3	Body Up	Lifting head and body off substrate
4	Move Toward	Moving slowly toward (any distance)
5	Nudge	Pushing intruder with the nose
6	Chase	Running or darting after in pursuit
7	Snap	Bite attempt, no contact
8	Bite	Bite <1sec
9	Bite Hold	Bite >1sec

Table 1.1. Aggression ethogram. Scores are ranked from lowest (1) to highest (9) in increasing aggression. Each movement was scored as a distinct event. Most of the salamander's movements are abrupt and distinct, enabling each event to be scored discretely. Level of aggression was quantified in four ways: 1) aggression score- the sum of acts times the score for each act, 2) adjusted aggression score- the aggression score divided by the number of times the intruder approached the resident, 3) number of snaps and bites- the sum of snaps+bites+bite holds, and 4) a qualitative assessment (each individual was delegated into one of two categories- low aggression= no behavior with a score above 4, or high aggression= any behavior with a score of 5 or above.

covering the egg masses were removed. A male or non-gravid female conspecific intruder was placed within one body length of a resident female. *Plethodon cinereus* are more aggressive toward unfamiliar individuals than familiar individuals (Jaeger and Peterson, 2002); therefore, to ensure that intruders were unfamiliar to the residents, individuals collected from distances greater than one kilometer apart were paired (Jaeger,2005). Intruders and residents were also matched by length and mass (as closely as possible) to minimize size asymmetry effects (Mathis *et al.*, 2000). Behavior of residents and intruders was observed for 15 minutes, using focal sampling.

Clutch Size and Sex of Intruder.- In early July 2005, clutches of brooding resident females were randomly manipulated into either small (4 eggs) or large clutches (10 eggs). All clutches contained at least one foreign egg (from an unfamiliar female). Females were monitored for ten days post-manipulation, to ensure that they were attending the altered clutches. Brooding mothers almost always accepted the egg(s) and provided care. If a female did not accept the foreign eggs, data were excluded from the analysis (in 2005, N=2; in 2006, N=1). There was no bias between small and large manipulated clutches in these failures. Non-brooding female residents were used as controls (N=24). Males and non-gravid females were used as intruders. Resident females (N=14) were initially paired randomly with either a male or female intruder. One week later, the same resident was paired with an intruder of the opposite sex.

The mean of the aggression scores for the two encounters was used in statistical analysis comparing females with small (N=7) and large (N=7) clutches

with one-tailed two-sample t-tests assuming unequal variance. Number of snaps and bites toward intruders for females guarding large and small clutches were analyzed with one-tailed two-sample t-tests assuming unequal variance. Aggression scores adjusted for the behavior of the intruder were also analyzed with one-tailed two-sample t-tests assuming unequal variance. Fisher's exact non-parametric tests were used to analyze the categorical data (behavior classified as either low aggression or high aggression). To examine whether females base their decision to guard on initial rather than manipulated clutch size, each female's initial (unmanipulated) clutch size (N=14) was regressed against total aggression scores.

To analyze the effect of the *sex of intruder,* aggression scores from the same brooding individuals in the *clutch size* experiment were used. Overall aggression scores, the number of snaps and bites toward male or female intruders, and aggression scores adjusted for the behavior of the intruder data were analyzed using one-tailed paired t-tests. Fisher's exact non-parametric tests were used to analyze the categorical data (behavior classified as either low aggression or high aggression). Four-way ANOVA and Tukey's Honest Significance Test were used to test for interactions between groups.

Clutch Age.- In summer of 2006, brooding females with their eggs were collected. Non-gravid females were also collected to be used as intruders. The early invasions were staged in late July when clutches were approximately four weeks old. Clutch age was based on comparison to the 2005 developmental photographs (Appendix B). Each female was tested again two weeks later for

the late encounters (N=17). To further confirm egg age at test date, age was backdated from the date of the first appearance of hatchlings. Development is 51-55 days (7-8 weeks) for this population at 20° C in the lab (personal observation, although Bachmann 1964 reported 60 days); an average of 53 days was used to calculate egg age. The early test occurred approximately 3-4 weeks pre-hatch (4 weeks after oviposition) and the late test occurred approximately 1-2 weeks pre-hatch (6 weeks after oviposition). A different female intruder was used in early and late encounters. Overall aggression scores, adjusted aggression scores, and number of snaps and bites for the early and late encounters for each female were compared using one-tailed paired t-tests. Fisher's exact non-parametric tests were used to analyze the categorical data (behavior classified as either low aggression or high aggression).

Body Size.- To assess the effect of body size of the resident, aggression scores and size measurements from the 2005 *clutch size* and 2006 *clutch age* experiments were used. Mass, total length, and snout-vent length (Petranka, 1998) and/or snout-leg length (Szuba *et al.*, 2002) of each female was measured at time of collection in spring and again at release in late summer (for brooding females this was after all eggs hatched). Spring and late summer measurements were averaged for use in statistical analyses. Log10 mass was regressed against total length. Mean mass, total length, and snout-leg length were each individually regressed against mean aggression scores. In addition, the ratio of log 10 mass to total length was used to calculate each female's relative body

size. Relative body size and the residuals were regressed against mean aggression score.

Additional statistics.- Additional comparisons made were: overall aggression scores, number of snaps and bites, latency to first aggressive act, and low versus high aggression for brooding residents versus controls (non-brooding residents), brooding residents versus intruders, and controls (non-brooding residents) versus intruders. Adjusted aggression scores were also compared for brooding residents versus controls. Differences in behavior scores for these comparisons were analyzed using t-tests assuming unequal variance.

Results

Many brooding females showed high tenacity in guarding their eggs, and were highly aggressive when an intruder was introduced. The initial behavior of most brooding residents was to guard (curl around) eggs. Next, the brooding mothers typically responded sequentially with head up, nudge, chase, and snap. In many encounters biting and even bite/holding occurred. In several encounters the brooding resident bit the intruder more than ten times, and in one encounter the brooding resident bit the intruder more than 21 times in the 15 minute tests. Most of these bites were directed at the tail of the intruder, although a few bites to the head and body were observed. In one instance, the resident bit and physically overturned the intruder. In a few cases, after 15 minutes of observations the brooding resident was still engaged in defensive behavior. In almost all encounters the non-brooding residents were stationary and were non-aggressive, and intruders usually attempted to escape or hide. In only one encounter did an intruder attack a resident.

Brooding residents were significantly more aggressive than non-brooding residents ($df=36$, $t=4.57$, $P<0.001$) in overall aggression scores (Table 1.2, Figure 1.1), and scores adjusted for the number of approaches by the intruder (Table 1.3). Brooding residents snapped and bit significantly more often than non-brooding residents ($df = 36$, $t = 3.2$, $P <0.001$) (Table 1.4). When the data were categorized into either low aggression or high aggression, Fisher's exact non-parametric test also showed significantly higher aggression in brooding versus non-brooding females (Table 1.5). Latency to first aggressive act was

	Residents vs. controls		Residents vs. intruders		Controls vs. intruders		Residents vs. residents		Residents vs. residents		Residents vs. residents	
							Clutch Size		Sex of Intruder		Clutch Age	
	BR	NBR	BR	I	NBR	I	Small 4 eggs	Large 10 eggs	F	M	Early 4 weeks	Late 6 weeks
N=	14	24	14	14	24	24	7	7	14	14	17	17
Mean Agg. score	84.7	3.6	84.7	10.0	3.6	2.6	83.9	79.6	93.1	70.4	117.8	270.4
St. err.	17.6	2.4	17.6	2.7	2.4	1.6	21.5	30.8	29.5	22.8	26.9	63.3
t=	4.57		4.19		0.33		0.11		0.61		3.4	
P=	**<0.001***		**<0.001***		0.37		0.45		0.27		**0.002***	

Table 1.2. Summary of overall aggression scores from behavioral observations. A * indicates a significant difference between groups. BR= Brooding residents, NBR= controls (non-brooding residents), I= Intruders

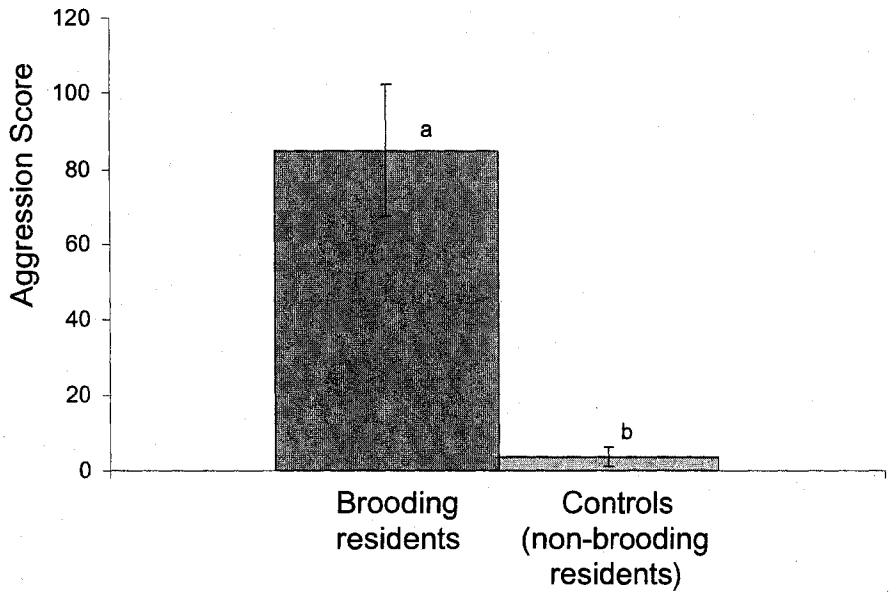

Figure 1.1. Aggression scores for brooding and control (non-brooding) females. Brooding females (N=14) were significantly more aggressive than controls (N=24) ($P<0.0001$). Mean ± se are shown.

	Residents vs. Controls		Residents vs. Residents Clutch Size		Residents vs. Residents Sex of Intruder		Residents vs. Residents Clutch Age	
	BR	**NBR**	**Small 4 eggs**	**Large 10 eggs**	**F**	**M**	**Early 4 weeks**	**Late 6 weeks**
Aggression score adjusted for # of approaches by intruder	25.7	3.5	26.1	25.4	30.9	20.4	46.0	104.5
St. err.	5.3	2.4	5.5	9.3	8.9	5.7	12.9	28.9
df=	37		13		27		33	
t=	3.6		0.07		2.19		2.4	
P=	**<0.001***		0.47		**0.02***		**0.01***	

Table 1.3. Summary of aggression scores after adjusting for the behavior of the intruder. Total aggression score of the resident was divided by the number of times the intruder approached the resident in the fifteen minute tests. A * indicates a significant difference between groups. BR= Brooding residents, NBR= controls (non-brooding residents)

	Residents vs. controls		Residents vs. intruders		Controls vs. intruders		Residents vs. residents		Residents vs. residents		Residents vs. residents	
							Clutch Size		Sex of Intruder		Clutch Age	
	BR	NBR	BR	I	NBR	I	Small 4 eggs	Large 10 eggs	F	M	Early 4 weeks	Late 6 weeks
N=	14	24	14	14	24	24	7	7	14	14	17	17
Mean # bites & snaps	4.1	0.5	4.1	0.1	0.5	0.4	3.6	3.9	4.7	2.9	3.3	10.8
St. err.	1.1	0.3	1.1	0.1	0.3	0.2	1.2	2.0	1.9	1.3	0.8	-2.8
t=	3.2		3.6		0.34		0.13		1.7		2.65	
P=	**<0.001***		**<0.001***		0.37		0.45		0.08		**0.009***	

Table 1.4. Summary of number of bites and snaps from behavioral observations. A * indicates a significant difference between groups. BR= Brooding residents, NBR= controls (non-brooding residents), I= Intruders

	Residents vs. controls		Residents vs. intruders		Controls vs. intruders		Residents vs. residents		Residents vs. residents		Residents vs. residents	
							Clutch Size		Sex of Intruder		Clutch Age	
	BR	NBR	BR	I	NBR	I	Small 4 eggs	Large 10 eggs	F	M	Early 4 weeks	Late 6 weeks
N=	14	24	14	14	24	24	7	7	14	14	17	17
# individuals low aggression	3	21	3	13	21	22	2	5	4	5	7	2
# individuals high aggression	11	3	11	1	3	2	5	2	10	9	10	15
P=	<0.001*		<0.001*		0.5		0.13		0.65		0.04*	

Table 1.5. Summary of results from Fisher's exact tests, categorizing behavior into one of two groups (low aggression = no score above 4, or high aggression= any score of 5 or above). A * indicates a significant difference between groups. BR= Brooding residents, NBR= controls (non-brooding residents), I= Intruders

significantly lower in brooding females as compared to non-brooding females (Table 1.6).

Brooding residents were also significantly more aggressive than intruders (df=26, t=4.19, P<0.0001) (Table 1.3, Figure 1.2); and they snapped and bit significantly more often than intruders (df=26, t=3.6, P<0.001) (Table 1.4). Fisher's exact non-parametric test also showed significantly higher aggression in brooding females versus intruders (Table 1.5). Latency to first aggressive act was significantly lower in brooding females as compared to intruders (Table 1.6).

Between non-brooding residents and intruders, no significant difference in overall aggression (df =46, t=0.33, P=0.37) (Table 1.2) or number of snaps and bites (df= 46, t=0.34, P=0.37) (Table 1.4) was detected. Fisher's exact non-parametric test also showed no significant difference between non-brooding females and intruders (Table 1.5). Latency to first aggressive act was not significantly different between non-brooding females and intruders (Table 1.6).

Clutch Size and Sex of Intruder.- The four-way ANOVA indicated that there were no significant interactions between clutch size, sex of intruder, and first versus second test (Table 1.7); therefore, one tailed t-tests assuming unequal variance were used to retain power. The four-way ANOVA indicated that there was a significant effect of individual; individual females behaved consistently regardless of clutch size, sex of intruder, or first and second test (df =12, P<0.001) (Table 1.7). There was a large variance in aggression scores for guarding females.

	Residents vs. controls		Residents vs. intruders		Controls vs. intruders		Residents vs. Residents Clutch Size		Residents vs. Residents Sex of Intruder		Residents vs. Residents Clutch Age	
	BR	**NBR**	**BR**	**I**	**NBR**	**I**	**Small 4 eggs**	**Large 10 eggs**	**F**	**M**	**Early 4 weeks**	**Late 6 weeks**
Mean Latency (sec)	**193***	**438***	**193***	**390***	438	459	213	205	272	128	**380**	**192***
St. err.	38.2	87.3	38.2	102.7	87.3	79.3	59.6	54.8	53.0	48.5	84.3	43.2
df=	9		10		14		11		14		10	
t=	2.6		1.79		1.76		0.09		1.6		2.12	
P=	**0.02***		**0.05***		0.43		0.46		0.08		**0.02***	

Table 1.6. Summary of latency to first highly aggressive act (score of 5 or above) from behavioral observations. Individuals that did not score 5 or above were excluded from the analysis. A * indicates a significant difference between groups. BR= Brooding residents, NBR= controls (non-brooding residents), I= Intruders

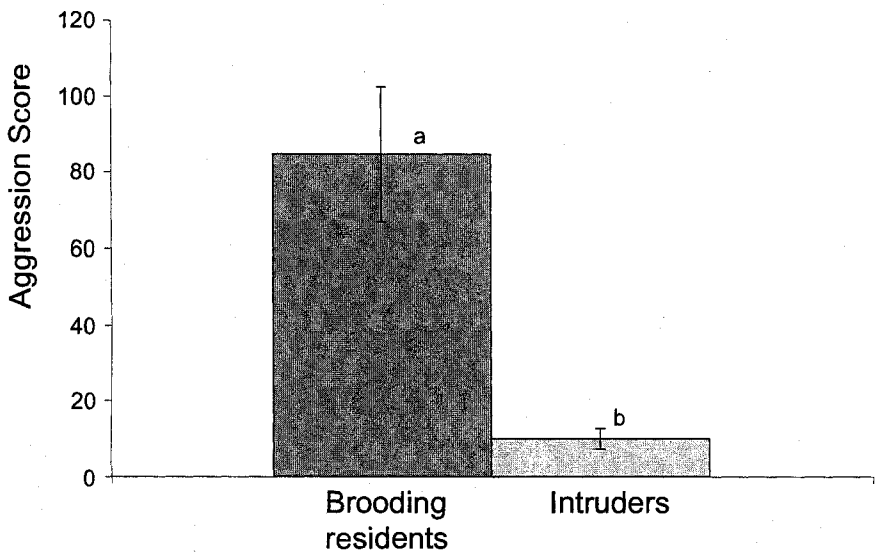

Figure 1.2. Aggression scores for brooding females and intruders. Brooding females (N=14) were significantly more aggressive than intruders (N=14) ($P<0.0001$). Intruder group includes both male and female individuals. Mean ± se are shown.

	Individual	Clutch size	Sex of intruder	Test order	Clutch size vs. test order	Clutch size vs. sex of intruder	Test order vs. sex of intruder	Clutch size vs. test order vs. sex of intruder
Sum of squares	837.7	3718.6	432.7	2334.8	1060.2	20.2	10251.1	2890.6
df	13	1	1	1	1	1	1	1
Mean-square	64.4	3718.6	432.7	2334.8	1060.2	20.2	10251.1	2890.6
F-ratio	8.7	0.31	0.03	0.19	0.08	0.00	0.85	0.24
P	**<0.0001***	0.58	0.85	0.66	0.77	0.97	0.37	0.63

Table 1.7. Summary of results from four-way ANOVA testing for interactions with individual, clutch size, sex of intruder, and test order as main effects. A * indicates a significant effect

Females were not significantly more aggressive when guarding small (4 eggs) or large clutches (10 eggs) (df = 13, t = 0.11, P = 0.45) (Table 1.3, Figure 1.3), even using scores adjusted for the number of approaches by the intruder (Table 1.3); nor was there a significant difference in number of snaps and bites (df=13, t=0.13, 0.45) (Table 1.4) Due to a small sample size and high variance, statistical power for these tests were low (0.22), however females exhibited high aggression when guarding either clutch size. Fisher's exact tests for clutch size also showed no significant difference in aggression when guarding large or small clutches (Table 1.5). The analysis of latency to the first aggressive act also showed no significant difference by clutch size (Table 1.6).

The smallest initial clutch size was four. The relationship between aggression scores and female's initial clutch size was negligible (R^2< 0.001, F= 0.06, P=0.94) (Figure 1.4). Females were not significantly more aggressive during the first encounter (81.4 ± 26.0) or the second encounter (82.1 ± 27.1) (mean aggression score ± standard error) (df = 27, t = -0.017, P= 0.49).

Females were aggressive toward both male and female intruders, with no significant difference in level of aggressive toward male or female intruders (df = 27, t = 0.61, P= 0.27) using overall scores (Table 1.2, Figure 1.5); although using the scores adjusted for the number of approaches by the intruder females were significantly more aggressive towards females (Table 1.3). There was a trend toward an increased number of snaps and bites directed at females (df = 27, t=1.7, P=0.08) (Table 1.4). Fisher's exact tests also showed no significant

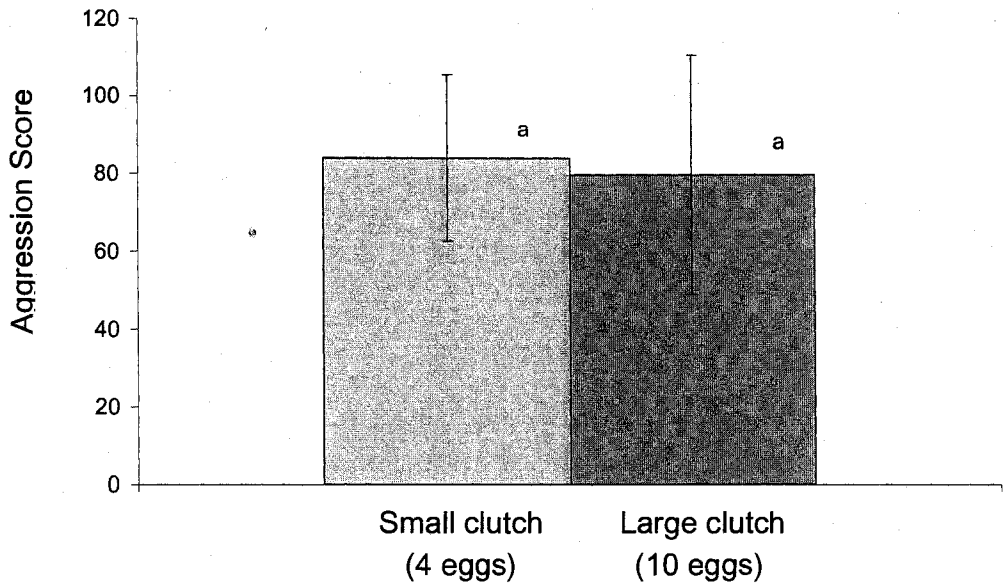

Figure 1.3. Aggression scores for brooding females with large or small clutches. There was no significant difference in aggression when guarding small (N=7) or large (N=7) clutches (t = 0.11, P= 0.45). Males and non-gravid females were used as the intruders. Mean ± se are shown.

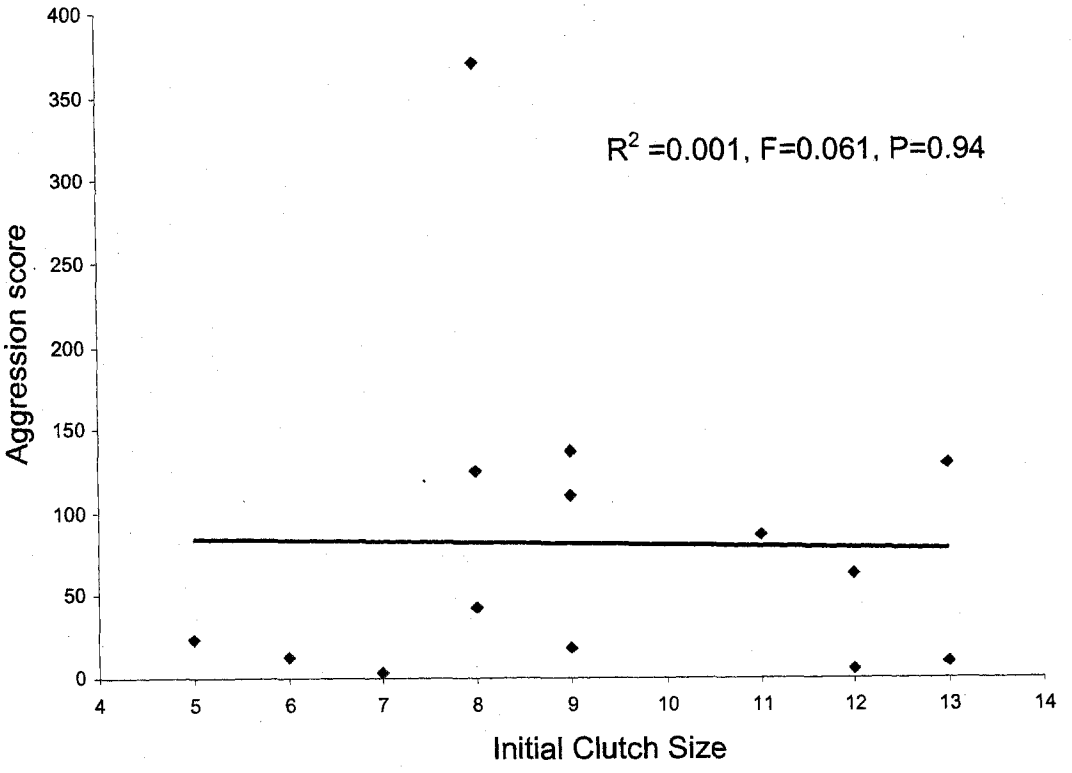

Figure 1.4. Influence of initial clutch size on aggression score. There was no relationship between female's initial (unmanipulated) clutch size and aggression scores (N=14). Mean aggression score for the two intrusions are shown

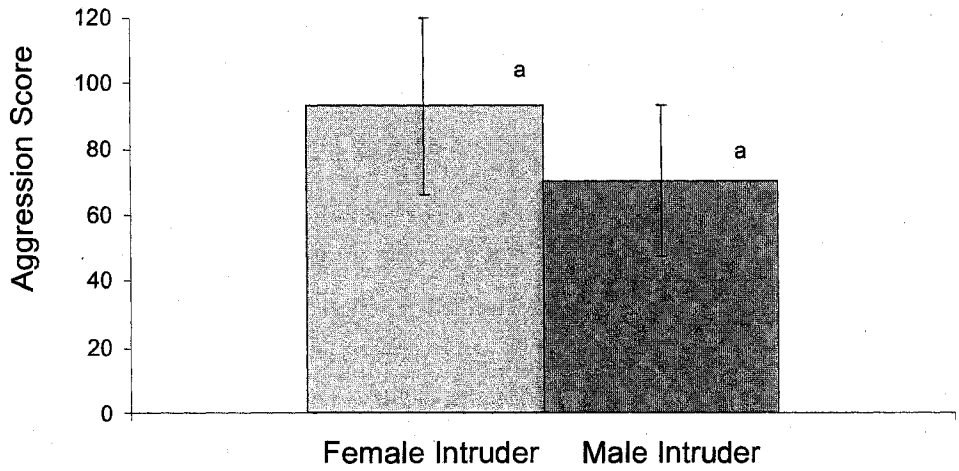

Figure 1.5. Aggression scores for brooding females for encounters with female and male intruders. There was no significant difference in total aggression when guarding against female versus male intruders (N=14) (t = 0.61, P=0.27). Individual aggression scores are from the same individual brooding females as for the *clutch size* experiment. Mean ± se are shown.

differences for sex of intruder (Table 1.5). There was a trend toward lower latency to first aggressive act directed toward males (Table 1.6).

Clutch Age.- There was a large variance in aggression scores for females guarding clutches in the *clutch age* experiment. Females guarding broods were significantly more aggressive when guarding older clutches (6 weeks old) than they were when guarding younger clutches (4 weeks old) ($df = 33$, $t = 3.4$, $P = 0.002$) (Table 1.2, Figure 1.6); scores adjusted for the number of approaches by the intruder were also significantly higher when guarding older clutches (Table 1.3). Significantly more females were also classified in the high aggression category when guarding late in the season (Fisher's exact test) ($P=0.04$) (Table 1.5). Latency to first aggressive act was significantly lower in females guarding the older clutches (Table 1.6).

Body Size.- In both years, over 50% of the variation in mass was explained by total length (in 2005, $R^2= 0.56$, $F= 16.3$, $P=0.001$) (in 2006, $R^2= 0.58$, $F= 20.9$, $P<0.001$) (Figure 1.7). Relative body size (of females and aggression scores were weakly correlated (in 2005, $R^2= 0.12$, $F= 1.8$, $P=0.19$) (in 2006, $R^2= 0.03$, $F=0.41$, $P=0.53$) (Figure 1.8); as were mass and aggression scores (in 2005, $R^2= 0.03$, $F=0.49$, $P=0.49$) (in 2006, $R^2= 0.005$, $F=0.07$, $P=0.78$), total length and aggression scores (in 2005, $R^2=0.04$, $F=0.51$, $P=0.49$) (in 2006, $R^2= 0.01$, $F=0.15$, $P=0.71$), and snout-leg length and aggression scores (in 2006, $R^2= 0.005$, $F=0.07$, $P=0.80$). Residuals of relative body size also did not explain level of aggression (Figure 1.9).

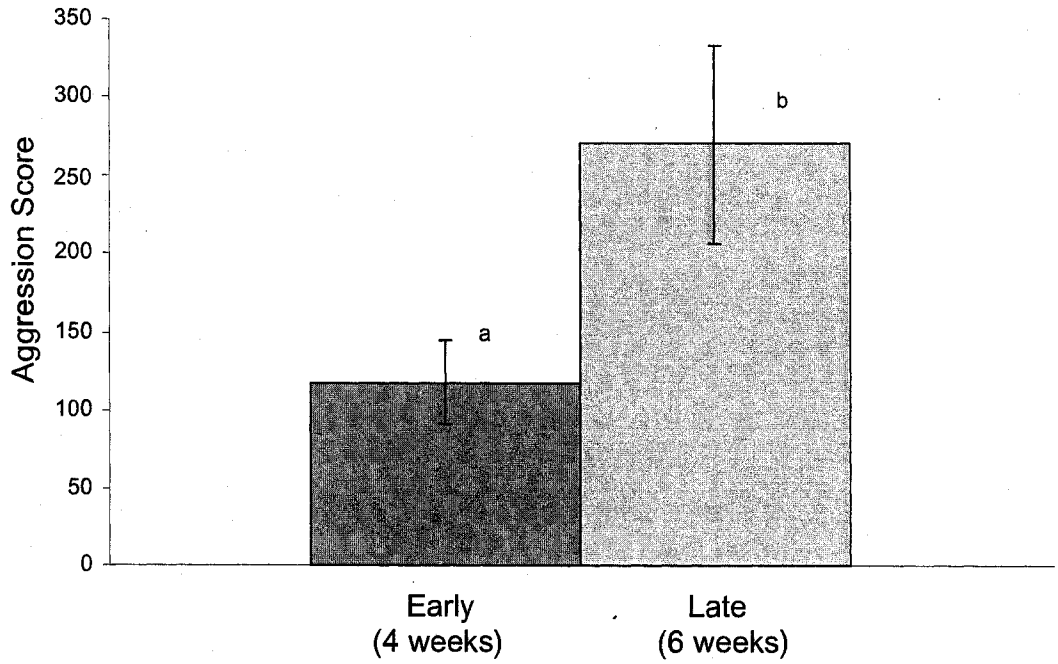

Figure 1.6. Aggression scores for brooding females with early and late clutches. Each brooding female was tested at 4 weeks post-oviposition and again at 6 weeks with a different non-gravid female intruder. Females were significantly more aggressive late than they were early in the brooding season (N=17) (t =-3.4, P=0.002). Note the magnitude of the y-axis. Mean ± se shown.

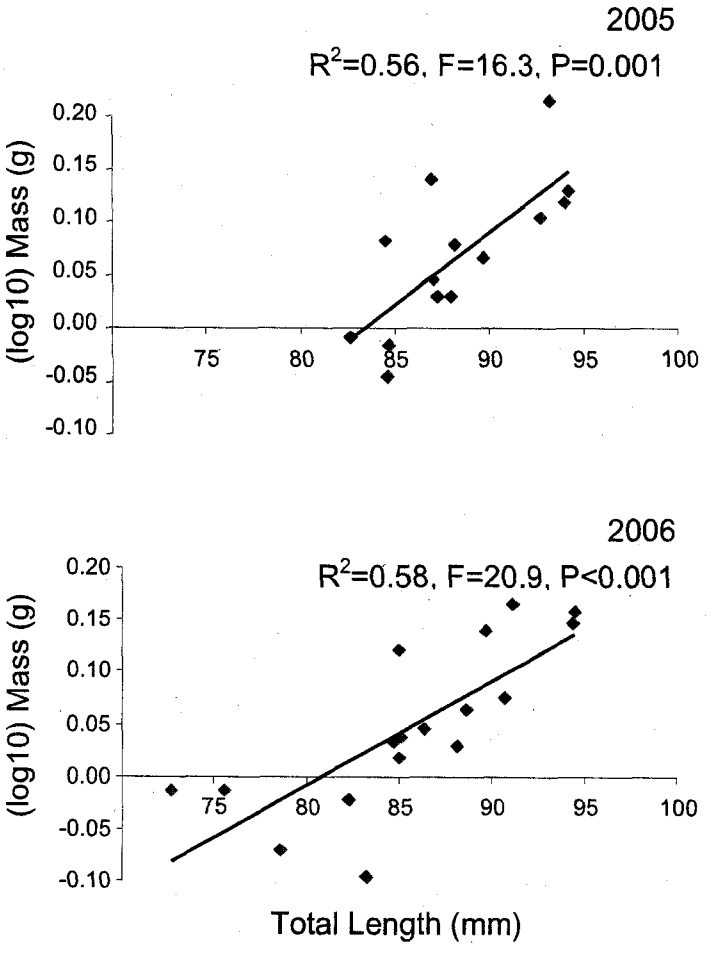

Figure 1.7. Regression of mass and total length. In both 2005 and 2006, a moderate relationship was detected between total length and mass. However, some females were simply long and slender, while others were short and fat. Log 10 mean mass and mean total length are shown.

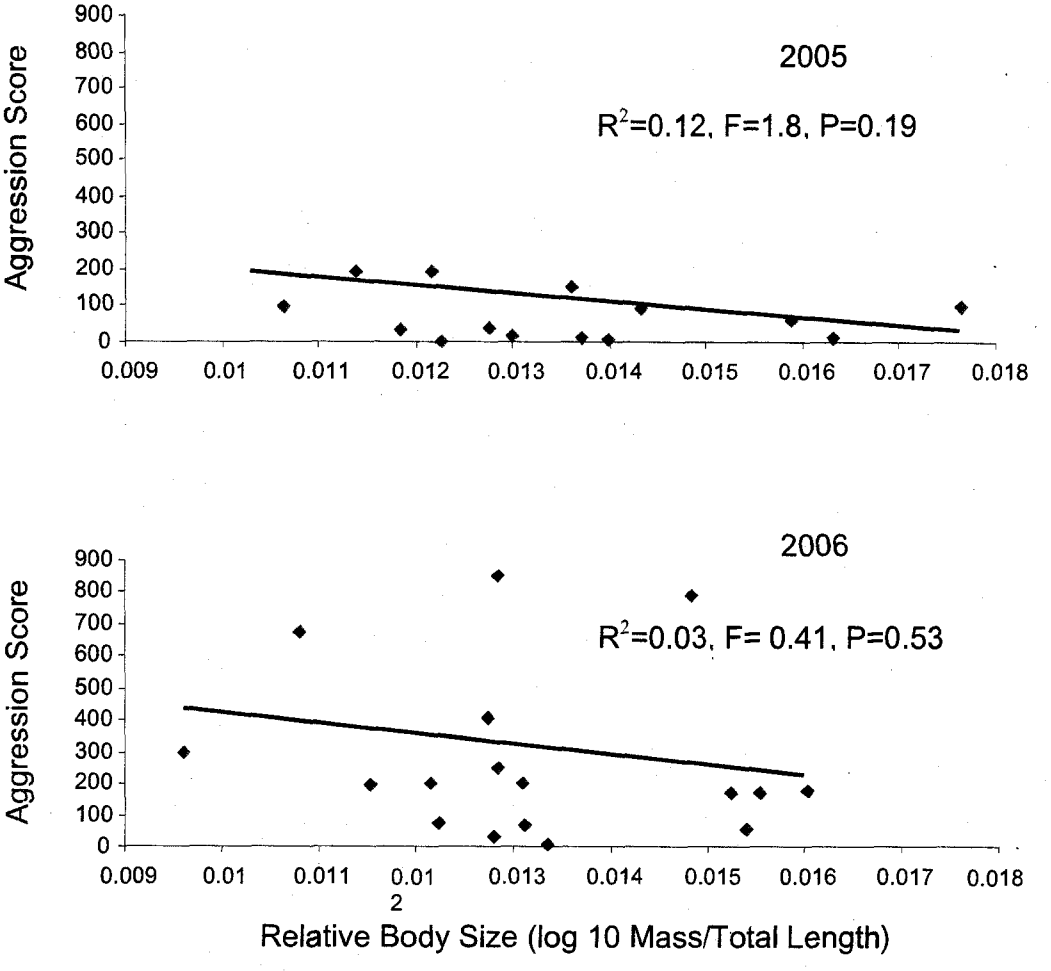

Figure 1.8. Regression of aggression and relative body size. No relationship was detected between relative body size (ratio of log 10 mass/total length) and aggression score in either 2005 or 2006. Mean aggression scores of the two encounters are shown.

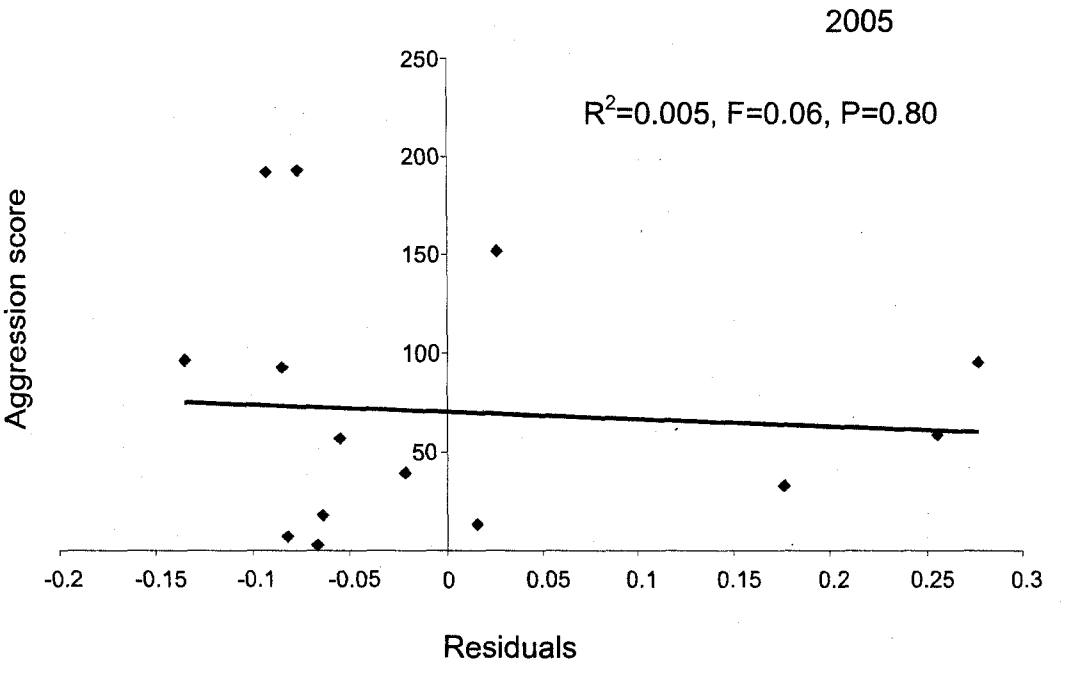

Figure 1.9. Regression of aggression and residuals of log10 mass/total length. No relationship was detected between residuals and aggression scores. Mean aggression scores of the two encounters are shown.

Discussion

In the *clutch size*, *sex of intruder*, and *clutch age* experiments, brooding female *P. cinereus* were aggressive in response to multiple conditions. Females aggressively defended both large and small clutches, were agonistic toward both male and female intruders, and elevated aggression to an extremely high level when defending late stage eggs. In addition, mothers of all sizes were willing to incur the risk of fighting to defend offspring and reduce the risk of cannibalism.

Attendance and nest guarding of eggs by *P. cinereus* increase the likelihood of offspring survivorship (Highton and Savage, 1961). Unattended Plethodontid eggs may quickly perish due to predation/ cannibalism, fungal infection, and/or desiccation (Forester, 1983). Attending females feed infrequently for the duration of the brooding period, which can be as long as two months. Thus, protection of each clutch of eggs represents an extremely high energy demand, and may be detrimental to the female's overall condition. One of the consequences of such a major depletion of energy may be the inability for females to reproduce every year. In addition, fighting may cause injury to body parts, especially to nasolabial grooves that aid in chemoreception. This can ultimately result in the inability to find food or mates and thereby the loss of future reproductive opportunities.

The elevated aggression scores in brooding residents as compared to non-brooding residents suggest that females are willing to incur greater risk when guarding offspring than when simply guarding resources such as food or dens. Researchers have not previously compared aggression between brooding (nest guarding) and non-brooding (territorial) female *P. cinereus,* although many

studies have clearly demonstrated territorial aggression in males and non-brooding females of this species (Jaeger *et al.*, 1982; Jaeger and Schwarz, 1991; Jaeger and Peterson, 2002). Data from my study may be interpreted two ways, either: 1) females are more aggressive when guarding eggs than they are when simply guarding a territory; or 2) non-brooding females kept in captivity do not recognize their housing as their own territory. The second explanation is less likely, since territories of males and non-brooding females are thought to be established in the laboratory after only five days (Nunes and Jaeger, 1989). A combination of results from the *sex of intruder* experiment, a comparison with other studies of territorial aggression in this species, and increased aggression in brooding versus non-brooding females suggest that females guarding eggs are more aggressive than those guarding a territory.

In contrast to the highly aggressive nature of brooding females, non-brooding females were far more passive. The passivity of the non-brooding females in this study diverges with field studies in which 74% of all territorial male and non-brooding female resident *P. cinereus* aggressively expelled intruders (Jaeger *et al.*, 1982). This difference in aggression between territorial non-brooding females in the Jaeger *et al.* study and the non-brooding females in my study may be a function of the time of year. My study took place in summer, while Jaeger *et al.* (1982) conducted experiments in spring. Perhaps non-brooding females are highly territorial in spring when searching for a suitable cover object in which to den. Differences in food quantity or quality between the two experiments might also explain these results; perhaps females in the field

experienced greater need for resource defense and therefore exhibited increased aggression.

Aggression was also significantly higher in brooding residents than intruders. In most encounters, intruders hid or attempted to escape by circling the container. This finding supports Bachmann's (1984) study in which 91% of brooding *P. cinereus* females, but only 5% of intruders were aggressive. Jaeger *et al.* (1982) also found that only 18% of male and non-brooding female intruders expelled territorial residents, residents were usually the victor. Many studies on various species suggest that residents are often successful in deterring intruders and are usually victorious in encounters between the two.

Females in this study aggressively defended clutches containing both familiar and unfamiliar eggs. In most of the manipulated clutches, females accepted and provided care for the foreign egg(s). Peterson (2000) found that *P. cinereus* demonstrates nest fidelity but not egg fidelity, suggesting that mothers may not be able to recognize their own eggs. Another study in which *Desmognathus fuscus* willingly surrogated unfamiliar eggs also suggests that some Plethodontids cannot recognize their own eggs (Forester *et al.*, 2005). On the other hand, *Desmognathus ochrophaeus* (Plethodontidae) can distinguish between their own eggs and unfamiliar eggs (Forester, 1983). Restricted to stream sides for nest sites, *D. ochrophaeus* females nest in close proximity to one another, and may have had selection pressure to develop a mechanism for egg discrimination. Since *P. cinereus* is not limited by the need to lay eggs near water, females may not nest as closely together. Territory recognition may be

sufficient for *P. cinereus*, rendering egg recognition capabilities unnecessary (Peterson, 2000).

Clutch Size.- This is the first study to directly compare clutch size with level of aggression in a nest guarding amphibian. Although aggression was high in many females, aggression scores during the defense of small (4 egg) versus large (10 egg) were not significantly different. Parental investment theory predicts that a large brood has greater reproductive value and should elicit greater parental efforts than a smaller brood (Williams, 1966; Winkler, 1987; Winkler and Wilkinson, 1988; Montgomerie and Weatherhead, 1988); therefore, parents should increase aggression when guarding a large number of eggs. One plausible explanation for the lack difference in aggression when defending large (10 egg) or small (4 egg) clutches is that a 4 egg clutch is still a considerable energetic investment. Each Plethodontid egg is sizable in comparison to the mother's body size. Even a small number of eggs represents a considerable energetic investment, and therefore this behavior may still be consistent with parental investment theory. From an evolutionary standpoint, the need to differentiate exact clutch sizes may have become an unnecessary adaptation. These data suggest that for *P. cinereus,* the benefits of defending a clutch consisting of as few as 4 eggs outweigh the costs/risks associated with this behavior. Initial (unmanipulated) clutch size also did not appear to have an effect on the level of aggression attained (i.e. larger initial clutches did not elicit increased aggression), lending further evidence that attendants do not base decisions to defend on clutch size.

Secondly, *P. cinereus* may not be able to distinguish the difference in quantity between a 4-egg clutch and a 10-egg clutch. Forced food-choice discrimination tests suggest that these salamanders can distinguish the difference between one and two prey items, and between two and three prey items; they preferentially selected the larger of the two numerosities. *P. cinereus*, however, failed to discriminate between three versus four prey items (Uller *et al.*, 2003). Given the salamander's rudimentary numerical capacity, more than two or three might just be considered to be "many". In that case, clutches of more than two eggs (most clutches consist of three or more eggs) should be guarded tenaciously if mothers always consider more than that a large clutch.

These data suggest that aggression does not increase with brood size in *P. cinereus*, and contrasts with research on other taxa that do alter behavior according to number of offspring. For example, male tree swallows, *Tachycineta bicolor*, chased intruders more often when defending large broods than when defending small broods (Lombardo, 1991). Pregnant mice (*Mus domesticus*) carrying large litters were more aggressive towards male conspecifics than those carrying small litters (D'amato et al, 2006). Care also increased with large brood size in male fathead minnows (*Pimephales promelas*) (Sargent, 1988). Few studies have been conducted to test whether aggression is positively correlated to clutch size, especially in amphibians. *P. cinereus* did not differentially defend the two clutch sizes (as did the birds, mammals, and fish cited above), emphasizing variations in parental strategies within the animal world.

A few studies have demonstrated that an organism's decision to defend or abandon a nest is based on clutch size. Yurewicz and Wilbur (2004) examined brood tenacity (the tendency to continue care rather than abandon a clutch) and found that female *P. cinereus* are more likely to abandon small clutches than larger ones. This seems to indicate that *P. cinereus* can distinguish differences in clutch size (or quantity of egg material) when deciding whether to attend or abandon clutches. If clutch size had been manipulated to fewer than four eggs, females may have been more likely to abandon rather than defend. My data suggests that once a mother has decided to attend a clutch, she may be likely to defend it regardless of the number of eggs it contains.

Clutch Age.- Brooding female *P. cinereus* guarded their eggs more aggressively late (6 weeks) than they did early (4 weeks) in development. This is consistent with parental investment theory, which predicts that an older brood is more likely to survive to hatching than a newly deposited brood, and therefore has greater reproductive value. (Winkler and Wilkinson, 1988; Montgomerie and Weatherhead, 1988). Literature addressing the question of clutch age and nest-defense is somewhat equivocal. Birds and rodents have most often been used to examine the effects of offspring age on aggression. My clutch age results are consistent with a study on tree swallows in which parents chased intruders more often when defending older offspring than when defending young offspring (Lombardo, 1990). Female mice also showed increased aggression during late pregnancy as opposed to early (D'amato et al, 2006). These studies provide

evidence for the ability of parents of various taxa to gauge brood value and make decisions accordingly.

The effect of clutch age on aggression is poorly understood in amphibians, and results from the few existing studies vary. In Bachmann's (1984) study, brooding *P. cinereus* did not increase aggression towards conspecifics late in the season, although neither size-matching nor familiarity of intruders was considered. On the other hand, my results are consistent with the findings of Forester's (1983) study in which *Desmognathus ochrophaeus* was significantly more aggressive later in the brooding season. If parents base their decision to increase aggression on age of the clutch, as Forester's (1983) study suggests, there may be a mechanism for recognizing egg development stage. When given a choice between thermally manipulated early and late stage eggs another Plethodontid salamander, *Desmognathus fuscus*, preferentially invested energy into brooding developmentally advanced eggs (Forester *et al.*, 2005). Remarkably, *D. fuscus* females even preferred to surrogate brood late stage eggs of a phylogenetically distant salamander species, *D. aeneus*, over early stage eggs of a more closely related species, *D. santeetlah* (Forester, 2005). The mechanism underlying this ability is yet unknown. These data suggest that *P. cinereus* females either: 1) have the ability to recognize egg developmental stage, 2) have a mechanism for calculating the amount of time passed since laying eggs, or 3) recognize the time in the season using external cues, and adjust care accordingly.

Although there was a significant increase in aggression in females guarding eggs at 6 weeks in the *clutch age* experiment, in the *clutch size* experiment there was no significant difference in aggression for tests that were staged one week apart. In the *clutch size* experiments, females were tested when eggs were at a similar developmental stage (4 and 5 weeks) as those tested early (4 weeks) in the *clutch age* experiment. This temporal discrepancy may explain why eggs were guarded more aggressively late rather than early in the *clutch age* experiment, but no increase in aggression was seen in the second test in the *clutch size* experiment. Within a one week gap (between weeks 4 and 5) egg development may not advance enough to elicit changes in care by the female, while within a two week gap the developmental changes that occur may be sufficient to elicit greater parental efforts. Females may recognize that embryos at an advanced stage in development (6 weeks) are better equipped to survive to hatching and therefore they may increase parental care at this stage.

Sex of the Intruder.- Although the various statistical tests used to examine the effect of sex of the intruder on level of aggression yielded differing results, collectively they indicate that brooding females were aggressive when guarding against both male and female intruders. Overall aggression scores and Fisher's exact tests both suggest that many females aggressively guarded against both sexes; although there was a trend towards more snaps and bites directed toward females, and significantly increased aggression toward females when the behavior of the intruder was considered. On the other hand, females tended to react more quickly with aggression toward males. Conspecifics of both sexes

may be encountered frequently in a natural setting, since population density in *P. cinereus* has been estimated as high as 1 individual / m^2 in some parts of its range (Maerz, 2001). Due to the high risk associated with aggression during nest guarding, this behavior should only be directed towards conspecifics posing a real threat to the eggs. My results suggest that females are willing to risk injury when confronted with both male and female intruders, therefore both sexes likely pose a threat.

Unfamiliar female *P. cinereus* are known to cannibalize nests (Highton and Savage, 1961, and Gibbons, *et al.*, 2003). Highton and Savage (1961) found that brooding female *P. cinereus* are aggressive toward cannibalistic conspecifics; however, the sex of the intruder was not documented in this study. Female *P. cinereus* selectively cannibalize the eggs and offspring of unfamiliar conspecifics more often than familiar offspring (Peterson, 2000; Gibbons, *et al.* 2003). These studies indicate that female intruders pose a serious threat to brooding mothers. It is less clear whether male *P. cinereus* also cannibalize eggs; although the remains of unidentified eggs have been observed in the stomachs of males, and cannibalism of juveniles has been recorded (Jaeger *et al.*, 2000).

Previous studies have not directly compared aggressive responses by an egg-guarding amphibian towards unfamiliar male and female intruders, although studies have addressed this question in territorial non-brooding or gravid females. Research on gravid *P. cinereus* females suggests that they are more tolerant of males than females (Horne, 1988). In another study, non-brooding (non-gravid) females also defended territories more strongly against female

intruders, and males defended territories more strongly against males (Lang and Jaeger, 2000). These two studies suggest that when eggs are not present, females have lower tolerance for female intruders; since an intruding female may be viewed as a competitor for both resources and mates, whereas an intruding male may be considered a potential future mate. My study suggest that when eggs are present, aggression towards both sexes is likely since intruders of both sexes may cannibalize eggs. These results imply that females may be more aggressive when guarding eggs than they are when simply guarding a territory.

Body Size.- Relative size (a function of both mass and length) may be an indication of the physical condition or resource holding potential of an individual. Increased body condition (larger reserve of energy) has a positive effect on hatchling survivorship (Horne *et al.*, 1990; Brumbaugh, 1998). Since mass may indicate a reserve of stored energy, it is logical to conjecture that parents with a greater mass should be able to defend the nest more effectively than those with less mass (Winkler, 1987). Contrary to my prediction, no correlation was found between any measurement of size (mass, total length, snout-leg length or mass/length ratio) and aggressive behavior. Neither mass nor length predicted level of aggression. In all cases, *body size* data tended towards an inverse relationship between size and aggression, although these relationships were not significant. There was a slight tendency for less long and less heavy brooding females to be more aggressive. The regression of body condition (log 10 mass/total length) against aggression score, also indicates a slight tendency toward smaller females defending more vigorously. My results are consistent

with Jaeger's (1982) study in which large territorial *P. cinereus* were not more likely to initiate attacks than small ones.

The relationship between body size and level of nest defense varies greatly by species. In some species a negative correlation exists between size and level of nest defense, in some species a there is no correlation between the two, and in others a positive correlation exists. For example, a negative correlation between weight and the magnitude of defense against predators was seen in female convict cichlids (*Cichlasoma nigrofasciatum*) (Galvani and Coleman, 1998). No correlation between size and site tenacity was seen in male smallmouth bass (*Micropterus dolomieu*) (Iguchi *et al.*, 2004). A positive correlation between female's body condition (weight) and nest defense was seen in female willow ptarmigans (*Lagopus lagopus*) (Martin and Horn,1993). Collectively, these studies suggest that in at least some species even relatively small individuals can successfully guard young.

Most females in my study were aggressive irrespective of size, suggesting that both large and small mothers are willing to incur costs of protecting their investment in the future generation. As these data show a lack of relationship between size and aggression, I suggest that for *P. cinereus*, age or experience may play a larger role than a parent's size in explaining variations in nest defense. An older or more experienced mother may simply curl tightly around the clutch to protect it, while a younger or inexperienced mother might actively pursue and attack an intruder. Perhaps past maternal experience may explain some of the variation. For instance, if a mother previously lost some or all of her

clutch to another salamander, she may increase aggression defending her present clutch. Few studies have examined the importance of age or experience on aggression during nest guarding.

Significant individual differences in behavior were demonstrated in these experiments. Individual brooding female *P. cinereus* behaved consistently (either passively or aggressively) under multiple conditions. This implies that additional factors such as age, past experience, individual variability, shy /bold "personalities", or dominance hierarchies may account for variations in aggressive behavior between individuals, and may explain consistent behavior within individuals. Recently, there has been a growing interest in the underlying physiological causes of similarities and differences in behavior among individuals. Several models have demonstrated that the development of animal personality can be adaptive, and that the evolution of personality is logically plausible (Bell, 2007).

These results emphasize behavior differences between species; and behavior within species may differ by reproductive status, time of the year, past experience, or even by personality. The large variance in aggression scores within the brooding female group may indicate that a resident's response was elicited by, and dependent upon, the behavior of the intruder. Adjusting aggressive behavior in accordance with each situation makes evolutionary sense, as it would be maladaptive to waste energy on aggression when an intruder simply wanders nearby without posing a threat to eggs. Individual females may behave more consistently under various conditions than previously

thought, bolstering other studies that lend evidence for repeatable temperament in animals. Many females were quite aggressive under several circumstances, indicating that for *P. cinereus*, each clutch of eggs (even those with as few as four eggs) represents a large investment in time and energy, and is therefore worth vigorously defending.

CHAPTER II

ENDOCRINE CONSEQUENCES OF NEST DEFENSE FOR BROODING RED-BACKED SALAMANDERS, *PLETHODON CINEREUS*

Abstract.- The challenge hypothesis seeks to explain variations in the temporal pattern of testosterone (T) in response to social conditions. It first described the endocrine response to a challenge by avian parental males and has since been extended to males from a wide array of vertebrate taxa, and to females of two taxa. The challenge hypothesis has not been investigated in amphibians. I used female red-backed salamanders, *Plethodon cinereus*, to examine the prediction that T will be elevated after a challenge in egg-guarding female amphibians. I challenged brooding *P. cinereus* by introducing a conspecific female intruder to a female guarding eggs, scored the resident's aggression, and took a tissue sample immediately post-encounter. To evaluate T level, I developed a novel non-lethal technique for extraction and assay of T using autotomized tail tissue. Tissue T was compared between challenged brooding females and controls (unchallenged brooding females). Challenged females had significantly higher T levels than unchallenged females, however regression analysis showed a weak inverse relationship between T concentration and level of aggression in individual salamanders. There was also a weak inverse relationship between females' relative body size and T concentration. To put my results in perspective, a seasonal profile of T for males and females was

determined. My data are consistent with patterns from seasonal profiles using plasma, therefore the use of tail tissue may be an alternative to lethal or invasive blood sampling, especially in endangered or threatened species with the ability to regenerate tissue.

Introduction

The challenge hypothesis (Wingfield *et al.*, 1990) originally described the androgen response to social stimuli during territorial defense, mating, and breeding by male birds. In polygynous male birds, high levels of plasma testosterone (T) are maintained throughout the breeding season; while in monogamous species, T has lower amplitude, elevates for a short period during territory establishment and mating, and then declines during the parental care period (Wingfield *et al.*, 1990). In these monogamous parental males, the challenge hypothesis predicts a surge in baseline T level in response to a territorial challenge to a nest or offspring (Wingfield *et al.*, 1990). High T seems incompatible with parental care and may have detrimental physiological effects (Cristóbal-Azakarate et al, 2006).

Since it was first proposed, the challenge hypothesis has been extended to many other vertebrates. However, large variations in androgen response exist among taxa, thereby making difficult a broad application of this theory to vertebrates. Hirschenhauser and Oliviera (2006) suggest that variations in patterns of breeding dispersal, social status, and group density may have confounding effects on androgen modulation in a particular system. Studies on many taxa have revealed differing baseline androgen levels during parental care, therefore comparing androgen responsiveness between species remains problematic. Although there is a general consensus that T is an important modulatory hormone for aggressive behavior, it remains unclear how the effects

of hormones on aggression (and vice versa) differ between classes of organisms. Hirschenhauser and Oliviera (2006) suggest a need for further direct quantitative analyses of the challenge hypothesis focusing on under-represented taxa.

Quantitative tests of the challenge hypothesis in amphibians have not been previously conducted. One likely explanation is that many amphibian species are promiscuous and non-parental (Hirschenhauser and Oliviera, 2006). Tests of several non-parental amphibians have revealed behaviorally induced androgen responsiveness (Burmeister and Wilczynski, 2001; Townsend *et al.*, 1991); whereas other studies revealed an absence of this phenomenon (Orchinik *et al.*, 1988; Houck and Woodley, 1995; Houck *et al.*, 1996). Parental care in its various forms occurs in at least 5% of anurans, and at least 20% of salamanders (some 72 species) (Crump, 1995). Seventy-nine percent of the salamanders that provide care are in the family Plethodontidae (Crump, 1995), and many of these species invest a great deal of time and energy into care. Yet, studies examining the role of T and other hormones on nest guarding and aggression in parental amphibians are scant, and none have directly tested the challenge hypotheses using simulated territory intrusions (STIs).

Although in most species males are more subject to social instability and must defend against intruders, in many cases females must also defend a mate or a nest. Studies on the challenge hypothesis in females of various taxa have yielded conflicting results. There have only been two studies on females thus far lending empirical evidence to support the challenge hypothesis. The challenge hypothesis has been demonstrated in female dunnocks (*Prunella modularis*)

(Langmore *et al.*, 2002), and in female cooperatively breeding cichlid fish (*Neolamprologus pulcher*) (Desjardins *et al.*, 2006). Studies of females of other species contradict the challenge hypothesis. For example, circulating androgens are not elevated in response to a STI in female song sparrows (*Melospiza melodia*) (Weatherhead, 1989), and California mice (*Peromyscus californicus*) (Trainor and Marler, 2001). Other hormones such as progesterone (P_4) and estradiol (E_2) may have a greater effect than T on maternal aggression in females of various taxa (Barfield, 1984; Davis and Marler, 2003; Rubenstein and Wikelski, 2005).

Study animals.- Female red-backed salamanders, *Plethodon cinereus* (Plethodontidae) are completely terrestrial and lay their eggs inside logs on the forest floor. They engage in lengthy maternal care, guarding and vibrating their eggs for 6-10 weeks (Bachmann, 1984) often without feeding; and may remain with the hatchlings for several weeks. Both males and females of this species are territorial, and their aggressive behaviors are well documented (Bachmann, 1984; Jaeger *et al.* 1982; Jaeger and Schwarz, 1991; Jaeger and Peterson, 2002). Its agonistic behavior consists of a series of threat postures (Jaeger and Schwarz 1991). Although researchers have extensively studied aggressive behaviors in red-backed salamanders, a hormonal basis for these behaviors has not been identified. In fact, little is known about the hormonal control of aggression in Plethodontids, although this Family contains more than half of all known salamander species.

Purpose.- My main goal was to quantify tissue levels of T and E_2 in *P. cinereus* immediately after a staged invasion of a nest cavity where a female was brooding her eggs. I challenged brooding residents by introducing a conspecific female intruder, and took a tissue sample immediately following the encounter to compare T and E_2 levels between challenged and control (unchallenged brooding) females. The tissue used to examine T and E_2 was obtained from salamander's tails. Tail autotomization, a defense mechanism against predators, is the process of self-amputation of a damaged or trapped appendage. I used autotomized tail tissue as an alternative to sampling from blood plasma, which may be lethal or painful. To validate the use of tail tissue and to establish baseline hormone levels, I quantified seasonal levels of T and E_2 for both males and females using radioimmunoassay (RIA). Novel aspects of this research include a direct test of the challenge hypothesis in parental amphibians, a seasonal hormone profile for female *P. cinereus*, and a technique utilizing tail tissue for RIA. While researchers have documented a seasonal profile of reproductive hormones in male and female salamanders from plasma for several species of caudates, baseline seasonal hormones for Plethodontid salamanders remain largely under-studied. Church and Okazaki (2002) reported seasonal T concentrations for male *P. cinereus* using blood plasma. This is only the second study to provide a seasonal hormone profile for male *P. cinereus*, and the first to examine hormones in tail tissue.

Materials and Methods

Collection and Husbandry.- Eastern red-backed salamanders were collected in 2006, by carefully hand searching in fallen logs in Durham, N.H. (latitude: 43.14 N, longitude: 70.93 W). Both striped and lead-backed (unstriped morph) individuals were used in this study; and only intact individuals with undamaged tails were used. Salamanders were weighed and measured for total length and snout-leg-length (Szuba *et al.,* 2000), then housed in covered plastic containers 19 cm x 13 cm x 9.5 cm. Approximately two centimeters of compressed soil covered the bottom of each container. Egg masses and females were placed under an inverted petri dish covered with a circular piece of brown paper towel (Brumbaugh, 1998). The towel helped to retain moisture and to keep the den dark. This technique also allowed observation by occasionally lifting the towel without disturbing the nest site. The containers were kept at 20°C, a 14L: 10D light cycle, and were misted occasionally to maintain a high relative humidity (Bachmann, 1984). Salamanders were fed white worms (*Enchytrae)* and wingless fruit flies, *Drosophila melanogaster*, several times per week.

Alternative Sampling Technique and Validation.- The technique used for sampling hormones in large salamanders has traditionally been to collect blood either by cardiac puncture (Church and Okazaki, 2002; Lecouteux *et al.*, 1984; Woodley, 1994; Zerani *et al.,* 1990) or decapitation (Specker and Moore, 1980). When salamanders are small, as is *P. cinereus*, blood pressure is extremely low, and samples are often taken from un-anesthetized and restrained animals

(Crespi, 2005). Although effective, these methods are probably painful and often lethal. Animal welfare advocates promote seeking alternatives to sacrificing experimental animals. Hence, there has been recent interest in finding non-lethal techniques for examining hormones, such as the use of ventral caudal scraping in the large spotted salamander (Homan *et al.,* 2003).

I sought an alternative technique using autotomization (induced self-amputation of a damaged or trapped appendage) of tail tissue instead of plasma for the radioimmunoassays. The use of severed tails may have less impact on the animals than traditional methods, and animals can be returned to the field. The use of tail tissue as a replacement for lethal or painful blood sampling may have implications for use on endangered or sensitive species when sacrifice of animals is not a viable option. To validate the use of tail tissue for detecting T and E_2, I established a seasonal hormone profile for both male and female red-backed salamanders to determine if relative hormone titres and seasonal patterns from the tissue are similar to those in the literature for plasma.

Seasonal Profile.- Male and female salamanders were field collected in spring, summer, and autumn of 2006. In April (spring), males (N=5), gravid females (N=5), and non-gravid females (N=5) were collected. In July (summer), males (N=5), brooding females (N=3), and non-brooding females (N=4) were collected. In September (autumn), males (N=5), and non-gravid non-brooding females (N=5) were collected. Sex and reproductive status of each individual was determined in the lab by "candling" since animals are nearly transparent and their gonads and eggs are easily visible when backlit (Gillette and Peterson, 2001).

Within two hours of field collection and sex/status identification, autotomization of tails was induced by pinching with a forceps approximately 6 to 9 mm posterior to the cloaca. Autotomized tails and salamanders were weighed, and the tail section was flash frozen on dry ice and stored at -20 degrees for use in the assay.

Challenge Hypothesis.- Female eastern red-backed salamanders with their egg masses (N=31) were collected for use as challenged females and unchallenged controls. Non-gravid females (N=28) were also collected for use as intruders. Females were allowed several weeks to acclimate to captivity, and to ensure that eggs were not abandoned. Between July 25 and 27, 2006, when eggs were approximately 30 to 40 days old, randomly designated brooding females were challenged (N=15) by introducing a non-gravid female intruder. Control brooding females were neither challenged nor disturbed (N=16). Observations took place between the hours of 8:00 p.m. and midnight, and were conducted under red light. Container covers and petri dishes covering the egg masses or dens were removed. The female intruder was placed within one body length of a resident female. Individuals captured from distances greater than one kilometer apart were paired to ensure that intruders were unfamiliar to the residents (Jaeger, 2005). Intruders and residents were also matched by length and mass, as closely as possible, to minimize size asymmetry effects (Mathis *et al.*, 2000).

Behavior of brooding residents was observed for 15 minutes, using focal sampling. Based on previously documented aggressive posturing behaviors

(Jaeger and Schwarz, 1991; Mathis *et al.*, 2000; Davis, 2002; Forester, 1983), and preliminary laboratory observations (Chapter I), an ethogram was developed for scoring the level of aggression displayed by brooding females during staged nest site invasions (Table 2.1). Aggressive behaviors were ranked increasing in intensity from one (guard eggs) through nine (bite hold). The rationale for this scoring system is based on the increasing effectiveness of each behavior in deterring an intruder, for example bites are more effective than nudges (Forester, 1983). All behaviors exhibited were scored according to the ethogram and summed. (See Chapter 1 where several alternative analyses for the behavior were explored for a validation of this scoring system.) This sum represented each female's aggression score for that encounter. Immediately following the encounter, each challenged brooding resident's tail was removed, weighed, and frozen, as previously described. Tails were also taken from undisturbed control females between July 25 and 27, 2006. Salamanders and hatchlings were kept in the laboratory until the hatchlings absorbed their yolk sacs and females' tails had grown back approximately 6 mm. All animals and their offspring were released to their original collection sites in September of 2006.

Extraction and Radioimmunoassay.- Each frozen tail tissue sample was finely minced with a razor blade, and placed in a 17 x 100 mm polypropylene tube. Phosphate Buffered Saline (8% NaCL; 0.003M NaH_2PO_4; 0.005M NA_2HPO_4; 0.12% Thimerosal) at pH 7.0 was added in a 4:1 ratio of buffer to tail mass. Tail/buffer mixture was homogenized for three minutes using a Kinematica CH-6010 Kriens-LU tissue homogenizer (Brinkman Instruments). The homogenate

Score	Behavior	Description
1	Guard Eggs	Moving to or curling around clutch
2	Head Up	Lifting head above neutral
3	Body Up	Lifting head and body off substrate
4	Move Toward	Moving slowly toward (any distance)
5	Nudge	Pushing intruder with the nose
6	Chase	Running or darting after in pursuit
7	Snap	Bite attempt, no contact
8	Bite	Bite <1sec
9	Bite Hold	Bite >1sec

Table 2.1. Aggression ethogram. Scores are ranked from lowest (1) to highest (9) in increasing aggression. Each movement was scored as a distinct event. Most of the salamander's movements are abrupt and distinct, enabling each event to be scored discretely. Level of aggression was quantified by the sum of acts times the score for each act

was centrifuged for 10 minutes at 4 degrees and 12,000 rpm, and the supernatant was decanted for extraction.

Extraction with ethyl ether followed, by adding 1.5 ml ether to each sample and vortexing for 10 seconds, then snap freezing the samples in an ether/dry ice bath for 10 seconds and decanting. This procedure was repeated two times, then the samples were dried under a stream of nitrogen in a water bath. Each dried tube was brought up to 500 µl for male samples and 200 µl for female samples with PG (PBS and 0.1% unflavored gelatin) buffer for use in the assay.

Testosterone titres were quantified by radioimmunoassay using the antiserum T3-125 Lot 338A, Batch 1502 (Esoterix) diluted to a final concentration of 1:750, following the methods of Sower and Schreck (1982). Tritiated (^{3}H) testosterone was added to each assay ($7.4x10^3$ DPM) for a final volume of 500 µl. Assays were left to equilibrate for 90 minutes. Bound and free T were separated by selective adsorption in charcoal dextran.

A standard curve was established from T concentrations of 1.95, 3.9, 7.8, 15.6, 31.3, 62.5, 125, and 250 µl. The sensitivity of the assay was 4 pg/ tube. Duplicate standards and duplicate pooled samples were run in each assay as positive controls. Unknowns were also run in duplicate. Testosterone assays were validated by running duplicate samples in different concentrations, dependent on the expected concentration of the unknown, to demonstrate parallelism with the standard curve. Samples were run in concentrations such that they fell onto the sensitive portion of the standard curve (between 7 and 60 pg/tube). Estimates for T concentration in the samples were calculated using

AssayZap (BioSoft, GB-United Kingdom). Extraction efficiency was calculated by adding a fixed amount (300 µl) of (^{3}H)T (approximately 2.2x10^4 DPM) to a tube with a mixture of buffer and chopped tail sample (4:1 ratio of PBS buffer to tail mass), This was extracted exactly as described above and then counted to quantify the extracted ^{3}H T. Extraction efficiency was 27.1%. Testosterone concentrations in the tissue were calculated from the mass of tissue taken, the concentration of sample, and the extraction efficiency . Intra-assay variation was 3.2%. Inter-assay variation was 6.7%. The range of antibody binding was 26.2% - 35.1%. The solvent blanks were negligible.

Estradiol titres were quantified by radioimmunoassay using antiserum at a final dilution of 1:85,000 with PG buffer (Sower and Schreck, 1982). Approximately 8.9x10^3 DPM tritiated (^{3}H) estradiol was added to each tube in a final volume of 500 µl. It was left to equilibrate for 30 minutes. Bound and free E_2 were separated by selective adsorption in charcoal dextran. A standard curve was established from E_2 concentrations of 0.49, 0.97, 1.95, 3.9, 7.8, 15.6, 31.2, and 62.5 µl. The sensitivity of the assay was 1 pg/ tube. Duplicate standards and duplicate pooled samples were run in each assay as positive controls. Unknowns were also run in duplicate. Duplicate samples in varying concentrations were run to demonstrate parallelism with the standard curve.

Statistical Analysis.- Seasonal hormone T titers were log transformed for analysis, and back-transformed for graphical depiction. Differences in male and female T levels (ng/g) between seasons were analyzed using ANOVA with Tukey's post-hoc Honestly Significantly Different test with SYSTAT (alpha= 0.05

for all statistical tests). Testosterone titers were compared between challenged and control females using a two sample t-test assuming unequal variance with Microsoft EXCEL. Testosterone level was regressed against aggression score for each brooding female. Testosterone level was also regressed against mass, total length, snout-leg length, and relative body size (the ratio of log10mass/ total length) of females using Microsoft EXCEL. Statistical outliers were determined using SYSTAT, and have been retained in all analyses.

Results

Seasonal Profile.- Seasonal tissue T titers for males differed significantly, increasing from spring to summer to autumn (df=2, F=44.75, P<0.03) (Table 2.2, Figure 2.1). Female tissue T levels were generally an order of magnitude lower than male T levels, except spring gravid females. Spring gravid females had significantly higher testosterone than any other female group (df=4, F=42.49, P<0.001) other female groups did not differ significantly between seasons (P>0.75 for all comparisons) (Table 2.2, Figure 2.2). Estradiol was below the level of detection in tail tissue in all seasonal female tissue samples even using the sensitized assay.

Challenge Hypothesis.- Challenged females had significantly higher T than unchallenged controls (0.233 ± 0.09 ng/g versus 0.056 ± 0.027 ng/g) (df=30, t=1.69, P-one tail=0.04) (Figure 2.3). The initial reaction of most brooding residents to the intruder was to curl around the egg mass. Brooding mothers typically responded with the behaviors: head up, nudge, chase, and snap. In many encounters biting and even bite/holding occurred. Consistent with previous behavioral studies (Chapter I), there was a large variance in aggression in the challenged females.

Testosterone and individual aggression.- There was a negligible correlation between aggression scores and concentration of tissue T (N=15) (R^2=0.03, F= 0.53, P=0.48) (Figure 2.4). Removal of the outliers did not result in either a

	Males			Females				
	Spring	Summer	Autumn	Spring gravid	Spring non-gravid	Summer brooding	Summer non-brooding	Autumn
N=	5	5	5	5	5	3	4	5
Testosterone (ng/g)	1.09	7.98	22.85	2.30	0.28	0	0.09	0.07
St.err.	0.27	1.63	2.37	0.23	0.18	0	0.09	0.07

Table 2.2. Summary of seasonal testosterone titers in tail tissue for males and females. Untransformed mean T concentration is shown

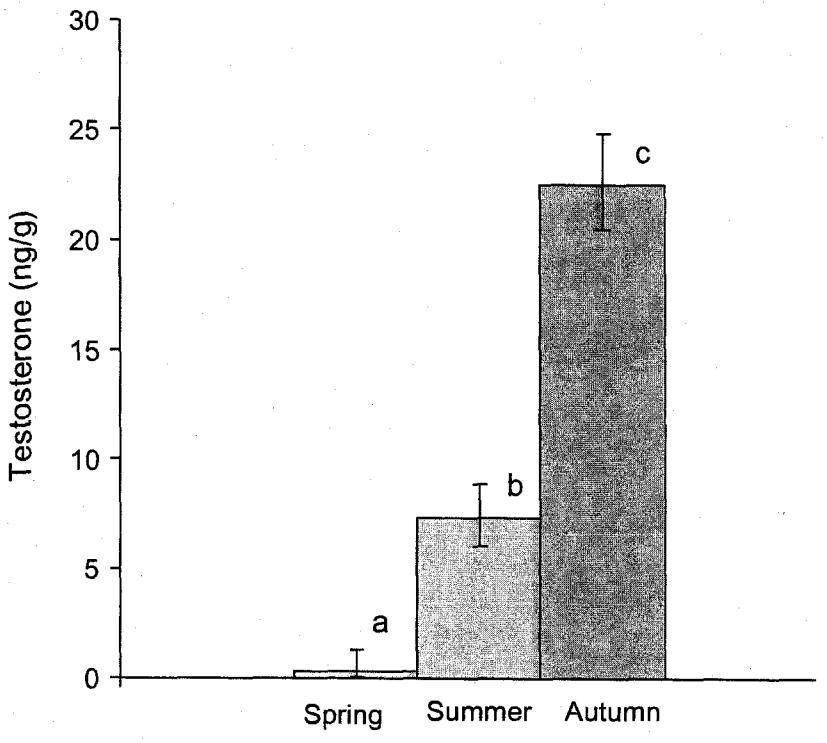

Figure 2.1. Seasonal testosterone profile for males. Male seasonal T titers differed significantly between spring and summer ($P=0.032$), spring and autumn ($P<0.001$), and summer and autumn ($P<0.001$) ($N=5$ for each group). Mean testosterone titers (back transformed) and standard errors are shown.

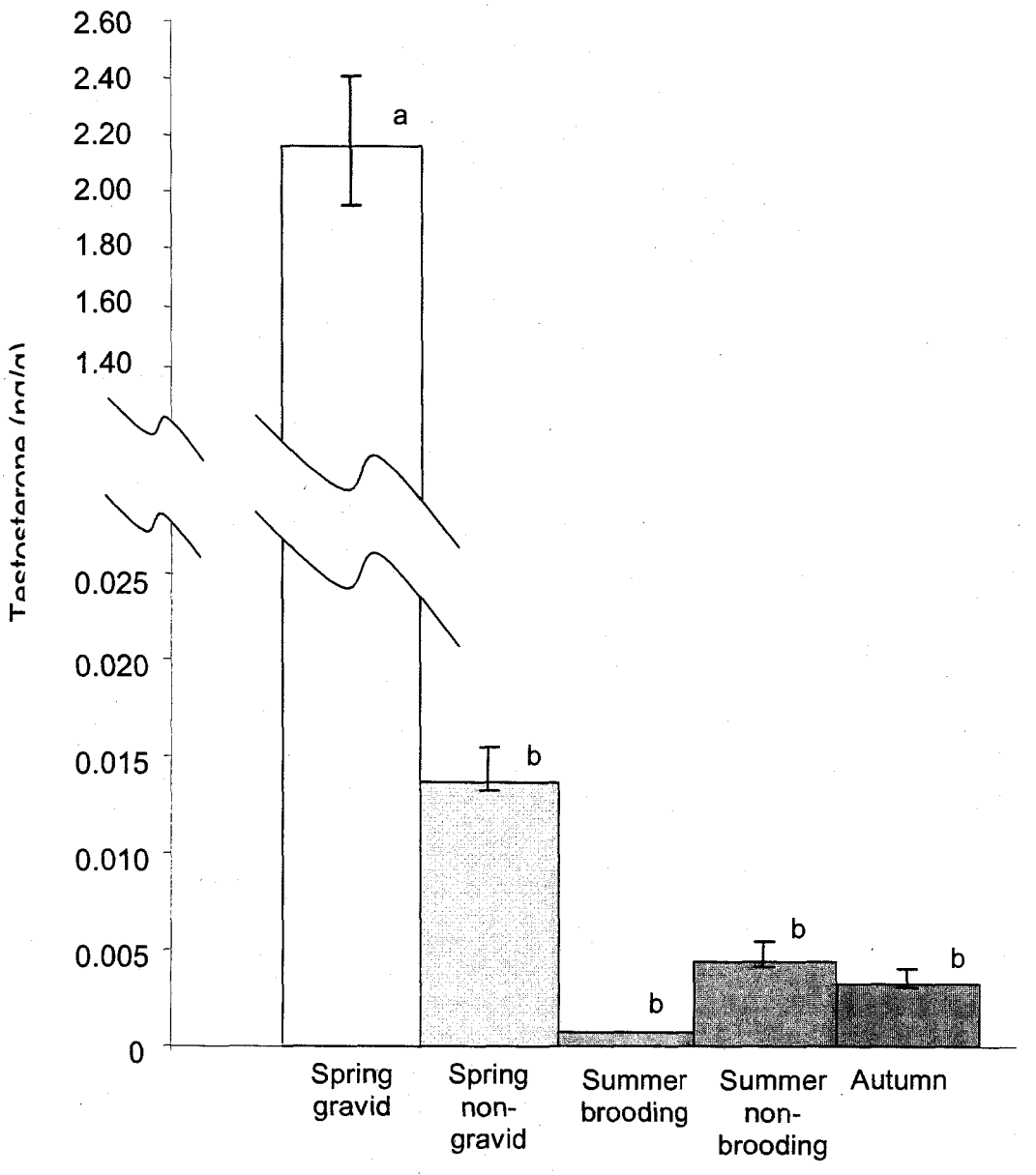

Figure 2.2. Seasonal testosterone profile for breeding and non-breeding females. Spring gravid females had significantly higher testosterone than any other group ($P<0.001$ for all comparisons). Spring gravid, N=5; spring non-gravid, N=5; summer brooding, N=3, summer non-brooding, N=4, autumn, N=5). Mean testosterone titers (back transformed) and standard errors are shown.

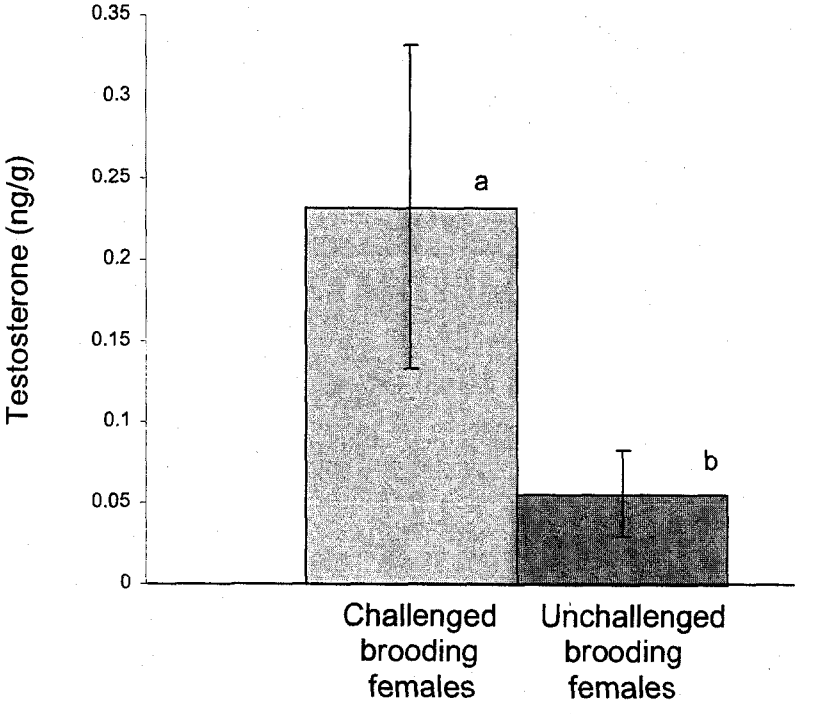

Figure 2.3. Testosterone titers for challenged and unchallenged brooding females. Challenged brooding females (N=15) had significantly higher tissue T than control (unchallenged) brooding females (N=16) (t critical one-tail=1.69, P-one tail=0.04). Mean testosterone titers and standard errors are shown.

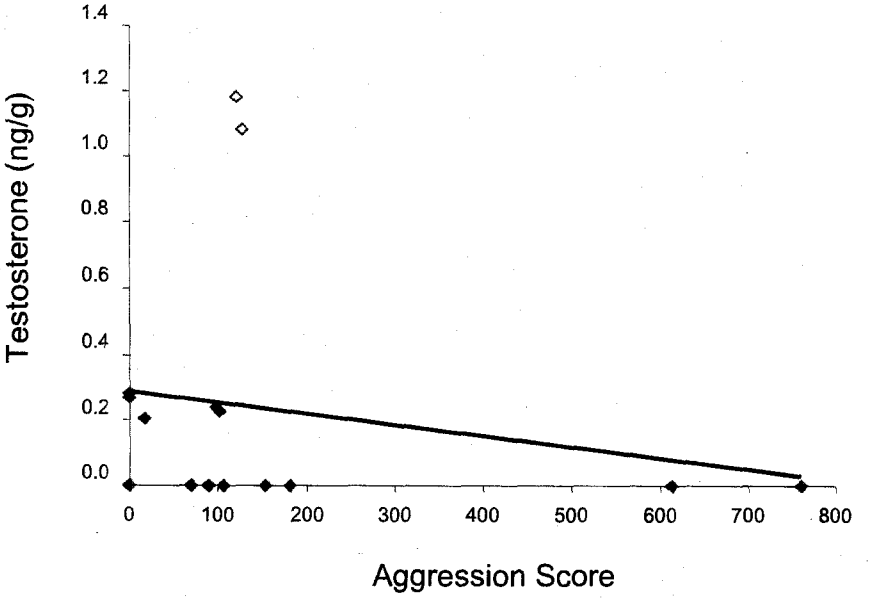

Figure 2.4. Regression of testosterone and aggression. There was a negligible correlation between level of aggression and T concentration in tail tissue of each challenged brooding female (N=15) (R^2=0.04, F= 0.53, P=0.48). Two of the most aggressive females had undetectable T. Removal of the outliers (open circles) did not result in a either a strong correlation or a positive relationship between individual aggression and T level (N=13) (R^2= 0.18, F = 2.54, P=0.14).

strong correlation or a positive relationship between individual aggression and T level (N=13) (R^2=0.18, F=2.54, P=0.14).

Testosterone and Body Size.- No measure of body size strongly predicted level of T, with or without the single outlier. There were weak negative correlations between each female's mass and T concentration in tail tissue with the outlier (N=15) (R^2=0.17, F=2.66, P=0.12) or without the outlier (N=14) (R^2=0.05, F=0.60, P=0.45); between each female's total length and T concentration in tail tissue with the outlier (N=15) (R^2=0.05, F=0.72, P=0.41) or without the outlier (N=14) (R^2=0.02, F=0.20, P=0.66); between each female's snout-leg length and T concentration in tail tissue with the outlier (N=14) (R^2=0.07, F=1.02, P=0.32) or without the outlier (N=14) (R^2<0.001, F<0.001, P=0.98); and between each female's relative body size (log10mass/total length) and T concentration in tail tissue with the outlier (N=15) (R^2=0.15, F=2.31, P=0.15) or without the outlier (N=14) (R^2=0.12, F=1.71, P=0.21) (Figure 2.5). There was a slight tendency in all cases towards smaller individuals having higher T. Two of the smallest individuals had elevated T.

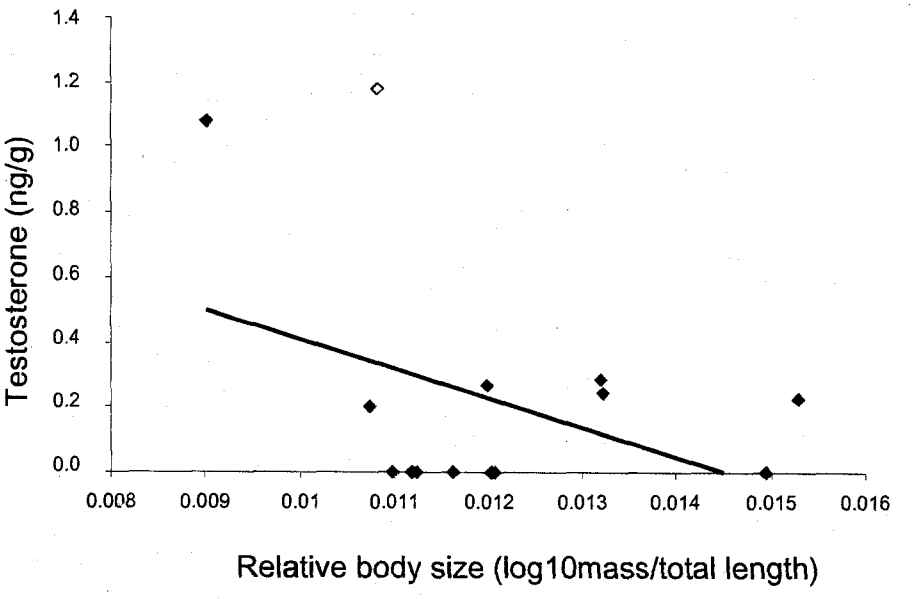

Figure 2.5. Regression of testosterone and relative body size. There was a weak negative correlation between each female's relative body size and T concentration in tail tissue with the outlier (open circle) (N=15) (R^2=0.15, F=2.31, P=0.15) or without the outlier (N=14) (R^2=0.12, F=1.71, P=0.21).

Discussion

Challenge Hypothesis in Amphibians.- Testosterone titers were significantly higher in the challenged versus the unchallenged brooding females, although the magnitude of these titers was small. Steroid hormones can induce physiological changes in minute concentrations, however it is impossible to determine whether the small albeit significant difference in T values between the two groups is biologically significant. Despite low T titers, I have demonstrated that, along with birds, mammals, fish, and reptiles (Hirschenhauser and Oliviera, 2006), parental amphibians also respond physiologically to a challenge by elevating T. There are many amphibian species that engage in parental care, yet the challenge hypothesis has not been examined in these species.

Testosterone is understood to be an important modulator of aggressive behavior, especially in males; however, studies of the effect of implanted T on aggression in male amphibians have yielded conflicting results. Researchers have demonstrated that T may modulate aggression in males of some anuran species (Houck and Woodley, 1995). Although high T is proposed to be incompatible with parental care in birds, T appears to have no effect on parental behavior in some amphibians. For example, male parental Puerto Rican tree frogs (*Eleutherodactylus coqui*) implanted with T pellets did not alter their brooding behavior, did not decrease nest fidelity, and did not increase abandonment or cannibalism of eggs (Townsend, 1991). It is not known whether T elevates in unmanipulated parental *coqui* in response to a territorial challenge.

Studies regarding the correlation of T and aggression are even less frequent for caudates than for anurans. Only one other study has explored this topic in a salamander. Male marbled salamanders, *Ambystoma opacum*, a non-parental species, did not elevate circulating plasma T levels in the presence of a male competitor as compared to solitary males (Houck *et al.,* 1996). Based on the existing literature for anurans, I expected wide inter-species variation (Houck and Woodley, 1995) in caudates; and I anticipated that the T response would likely differ in salamander species with different life histories and reproductive strategies. Therefore it was not surprising that hormone responses for parental female *P. cinereus* differ from non-parental male *A. opacum.* During breeding male *A. opacum* may have elevated baseline androgens which may preclude the need to elevate T. In a densely populated terrestrial egg-laying species such as *P. cinereus*, many intrusions to the nest are likely from both predators and conspecific cannibals, precipitating a need for a response from the guarding parent. Red-backed salamander eggs are also few (3-15) and large in proportion to the female's mass, and females reproduce only biennially. Such a large investment in a current clutch may result in the need for increased responsiveness, modulated by hormones such as T.

Challenge Hypothesis in Females.- My study is only the third to directly demonstrate an androgen response to a challenge in a female. Dunnocks, birds with a flexible mating system including facultative polyandry, provide support for the existence of the challenge hypothesis in females (Langmore *et al.*, 2002). In cooperatively breeding cichlid fish (*Neolamprologus pulcher*), females were

highly aggressive and had significantly increased T in response to a simulated intrusion to their breeding territory (Desjardins *et al.*, 2006). The Desjardins *et al.* study is the first to provide evidence of androgen responsiveness in a challenged female teleost fish, while mine is the first to do so in a female amphibian.

Studies of the effects of hormones such as T, E_2 and P_4 on aggressive behavior in females show large variations among taxa; however, the methods employed in these studies have been as widely varied as the results. Several studies of manipulated female mammals implicate T as a modulator of aggression. For example, ovariectomized T injected female rats showed increased aggressiveness over controls (Van de Poll *et al.*, 1988). In the cichlid fish (*Aequidens pulcher*), T increased agonistic behavior in females while estradiol decreased it (Munro and Pitcher, 1985). Ovariectomized female mountain spiny lizards (*Sceloporus jarrovi*), were less aggressive than free-living females, and implanting T pellets restored aggressive behavior in response to an intruder, suggesting a role of T on aggression (Woodley and Moore, 1999).

Yet other studies indicate a larger role E_2 and/or P_4 for than T for modulating aggression. In female rats and hamsters, E_2 and P_4 may suppress maternal aggression (Barfield *et al.*, 1984). Estradiol, rather than T, is also correlated with increased territorial aggression in female leopard geckos (*Eublepharis macularius*) (Rhen and Crews, 2000). In a direct test of the challenge hypothesis, female marine iguanas (*Amblyrhynchus cristatus*) elevated E_2 and P_4 (and not T) immediately following an aggressive encounter (Rubenstein and Wikelski, 2005). This supports the challenge hypothesis, but suggests that in

female iguanas T and perhaps P_4 may be rapidly aromatized to E_2 during aggressive interactions. This hypothesis merits further study in other female vertebrates. Although there is a general consensus that the hormones T, P_4, and E_2 modulate aggression in females; additional tests that include baseline seasonal and reproductive hormone levels as well as hormonal responses to STIs are required to further examine the challenge hypothesis in females.

Testosterone and Individual Aggression.- Although as a group the challenged brooding females had increased T compared to the control (unchallenged brooding) group, each individual female's aggression was negligibly correlated to her T level. Some females were very aggressive without greatly increased T, while other females were completely non-aggressive yet T was elevated. Females responded physiologically to an invasion of the nest, but this spike in hormones did not necessarily result in aggressive behavior. Testosterone may not be correlated to level of aggression for several reasons. First, a physiological response does not necessarily stipulate that a behavioral response will follow (i.e. a surge in T may prepare an animal to fight, but does not necessarily predict that a fight will occur). Second, the behavior of the brooding resident may depend upon the behavior of the intruder. Therefore, if the intruder hid or kept at a distance, the resident may not have escalated aggression. Females were physiologically prepared to defend their eggs, but only became aggressive if the intruder actively threatened the eggs. Third, other factors may account for individual differences in aggression such as the age of the mother, past experience, or temperament differences between individuals.

When examining the challenge hypothesis, it is important to consider subtle differences in experimental design, which may account for large differences in hormonal responses (Goymann *et al.*, 2007). For example, animals may respond differently to a decoy versus a live intruder, or to the posture of the opponent, the size of the arena, or the position of the opponent in the arena. In my experiment, preliminary observation in a large arena indicated that the arena size used in this study was adequate; resident females did not usually begin aggressive posturing until the intruder was in close proximity (within one body length) and did not pursue an intruder beyond 14 cm. Also, other studies used a smaller arena (15 cm in diameter) to test maternal aggression in salamanders with good result (Forester, 1983). Use of a model or a live albeit secured intruder, as opposed to a live free-ranging intruder may decrease the variance in behavior of the brooding residents, as the intruder's behavior would then be somewhat better regulated although not natural.

Testosterone and Body Size.- I report weak relationships to all measures of size (mass, total length, snout-leg length, or mass/total length) with level of T following a challenge. Likewise, I previously found a weak correlation between body size and level of aggression in individuals (Chapter 1). My data support similar findings for *P. cinereus* in which a negligible relationship was seen between T and snout-vent length, tail length, or mass (Church and Okazaki, 2002).

Seasonal Profile.- I was able to establish a seasonal profile of T from tail tissue in both male and female *P. cinereus*. Although it is impossible to directly

compare T values derived from tissue to those derived from plasma, my seasonal hormone profile for both male and female salamanders show patterns consistent with existing profiles from plasma in the literature. For males, the seasonal T pattern is similar to patterns of other Plethodontids from plasma, in which T is elevated in autumn. In *P. cinereus,* T peaked in autumn, and was lowest in May-July (Church and Okazaki, 2002). Woodley (1994) also examined T levels in two Plethodontid species, *Desmognathus ochrophaeus* and *Plethodon jordani.* Data from both species indicate that plasma T is maximal in the autumn, drops in spring, and is closely associated with male mating behavior (Woodley, 1994). The pattern was also quite closely matched to *Ambystoma tigrinum,* in which plasma T also spiked in September-October, was intermediate in July-August, and was lowest in March-June (Norris *et al.*, 1985). In the male crested newt (*Triturus carnifex*), plasma T profile is similar, but also remained high over the autumn/winter breeding months, and decreased around April (Zerani *et al.*, 1991). I was unable to ascertain winter values in my study for comparison, as *P. cinereus* hibernate. Studies of two other unrelated northern temperate salamander species, *Pleurodeles waltl* (Garnier, 1984) and *Triturus carnifex* (Zerani *et al.,* 1991), reveal a similar pattern of increased plasma T in autumn for males. My results are also consistent with another study using tissue rather than plasma to examine seasonal T levels. In the fire salamander (*Salamandra salamandra*) T in testicular tissue was highest in autumn, moderate in winter, and lowest in summer (Lecouteux *et al.,* 1995).

However, differences in male seasonal androgen patterns exist between caudate species. In male mudpuppies (*Necturus maculosus*), individuals varied greatly, but no significant difference in plasma T was seen between months or seasons (Bolaffi and Callard, 1979). In contrast with terrestrial breeding salamanders with internal fertilization (such as Plethodontids), a pond-breeding salamander with external fertilization, *Hynobius nigrescens*, had a very high peak of T in March corresponding to the peak breeding season (Hasumi *et al.*, 1993). The general trend of annual androgen fluctuations for male caudates is that T seems to correspond with mating (Houck and Woodley, 1995). In most male caudates, studies of seasonal hormones reveal a definite seasonal cycle, however variations in reproductive hormones are likely to be a function of different life histories and mating strategies.

This study provide the first data for seasonal T in female *P. cinereus*, since many studies evaluating the seasonal pattern of hormones in females have examined E_2 or P_4 rather than T. Competition for a suitable nesting territory in which to lay eggs may explain why T is relatively high in gravid females. My study supports results from another salamander species, *Salamandra salamandra,* in which plasma T, E_2, and P_4 were also significantly higher in gravid females than in non-gravid females (Degani *et al.*, 1997). Available information about female hormones suggests that seasonal reproductive patterns vary widely between species. In Spanish ribbed newts (*Pleurodeles waltl*), T in females is lower than in males but remains at higher levels than E_2 throughout the reproductive cycle (Garnier, 1985). In female mudpuppies (*Necturus*

maculosus), just as in males, high variation existed between individuals and no significant monthly difference in plasma T was seen (Bolaffi and Callard, 1979). In the female crested newt (*Triturus carnifex*), plasma T was high during the reproductive period, with T level peaking in February-March, and then dropped for the rest of the year (Zerani *et al.*, 1991).

Caution should be used when comparing seasonal hormone profiles in species with vastly different reproductive behaviors. For example, it may not be useful to compare hormone profiles from female pond breeding caudates like *Triturus carnifex* with those from completely terrestrial species such as *P. cinereus*. Pond-breeding females have only one chance to mate per year, while terrestrial females can obtain a spermatophore throughout autumn and spring, and can retain sperm for some time before ovulation and fertilization occur. Differences in sampling techniques may account for some of the subtle differences seen in the aforementioned studies. Most of the female salamanders for which a seasonal hormone profile is available do not demonstrate parental care; therefore, it remains unclear what effects parental care may have on circulating hormone levels or vice versa.

In my study, E_2 was below the level of detection using extraction from autotomized tails. In contrast, Bolaffi and Callard (1979) reported high blood plasma levels of estradiol in the mudpuppy (*Necturus maculosus*). There are two possible explanations for this difference in E_2 levels between the two species. First, the reproductive hormones in *Necturus*, a uni- or bi-parental aquatic salamander, may differ vastly from those in *Plethodon*, a terrestrial salamander

with extended female care. Second, E_2 may be present, but in concentrations below the level of detection when using tail tissue. An E_2 RIA for female *P. cinereus* using blood plasma would be necessary to address this question.

Technique.- This study demonstrates the use of autotomized salamander tail tissue as an alternative to blood plasma for detecting steroid hormones using RIA. Salamander tails were easily severed by pinching with forceps; and enough tissue was available from even small individuals for use in the assay. In some individuals severed tails weighed little as 0.15 grams. One limitation of such a small amount of tissue is the inability to repeat assays multiple times. In larger individuals, where severed tails weighed 0.20 g or more, enough material was available for multiple repeated assays of the same sample. If repeating similar tests on small salamanders, tails could be severed more closely to the cloaca than 6-9mm to gain more tissue for the assay. I also recommend repeating this technique on other large salamander and lizard species that can regenerate lost tissue.

Although tail autotomization is widespread in both lizards and salamanders, there is little agreement about its long term effects. The effects of tail autotomization on reproduction, mating, resource holding potential, escape from predators, etc. have been the subject of a multitude of studies. The results of such studies vary tremendously. Variables such as species differences and amount of tail loss preclude making broad generalizations about the detrimental effects of autotomization (Bernardo and Agosta, 2005). In *P. cinereus,* the tail comprises an estimated 20-35% of the total mass of an individual. Removal of

females' tails is thought to reduce internal energy stores, which may ultimately manifest in a decreased amount of energy available for vitellogenesis (yolk deposition) and potentially decrease production of future ova (Yurewicz and Wilbur, 2004). However, in a direct test of the effects of autotomization, brooding success was not reduced in tailless versus control mothers. Yurewicz and Wilbur (2004) suggest that, at least for *P cinereus,* fat reserves from tail tissue are not critical for successful brooding to occur. After several empirical tests on *Desmognathus ocoee* and *Desmognathus carolinensis* salamanders, Bernardo and Agosta (2005) found no evidence of complete reproductive inhibition as a result of tail loss. My research suggest that for small species, the use of autotomized tail tissue as an alternative to sacrificing experimental animals may be appropriate for some hormone assays, since at least some species of salamanders can survive and reproduce post-procedure.

Disadvantages of extracting steroids from autotomized tail tissue include limited amounts of tissue available for assay, and poor extraction efficiency. My tail tissue assay yielded an extraction efficiency of only 27%, yet low intra-assay (3.2%) and inter-assay (6.7%) coefficients of variation suggest that steroids can be reliably compared between individuals using this technique. A plausible explanation for low extraction efficiency is that T is lipophilic and hydrophobic. Tails tissue is comprosed of significant levels of fat, therefore the T may become trapped in residue after the homogenate is centrifuged (i.e. the pellet). In addition there is an abundance of a glue-like substance emitted by the autotomized tail, presumably produced as a deterrent to predators. Preliminary

chromatography of the viscous material revealed that it consists mainly of large protein molecules (unpublished data). This substance may bind up hormones in tail tissue, impeding the ability to extract them with higher efficiencies. All salamanders undergoing the autotomization procedure survived. After about a month, the captive females' tails had regenerated approximately 5 mm. I posit that this technique may have future applications when lethal or painful techniques may not be appropriate, such as for acquiring reproductive data about threatened or endangered species.

CONCLUSIONS

Parental care has evolved independently multiple times in vertebrate lineages, and occurs in approximately twenty percent of salamander species (Ng and Wilbur, 1995). In some salamander species, females produce large eggs and provide guarding of eggs and even neonates. For parental care to be adaptive, parents must balance the interest of their current offspring against their own survival needs and opportunities for future reproduction. Parental investment describes any parental behavior that both: 1) benefits offspring and 2) threatens future reproduction of parents (Clutton-Brock, 1991; Trivers, 1972). Nest guarding behavior fits both of these criteria since it enhances survival of offspring, but is energetically demanding, decreasing time for foraging and mating, and can result in injury or death of the attendant. As such, parents must have the ability to interpret the relative reproductive value of current offspring and must be able to evaluate the level of a given threat to the nest. These evaluations should help a parent decide whether and how strongly to defend, or whether to abandon offspring. This study examined several external cues that influence aggression during nest guarding, as well as the internal (endocrine) status of challenged brooding females.

Although some salamanders exhibit parental care, especially members of the family Plethodontidae, only a handful of studies have examined aggressive

behavior during nest guarding. Female *P. cinereus* devote an enormous amount of time and energy attending eggs, reduce feeding for up to two months, and risk injury to assure survivorship of their progeny. They tenaciously guard their eggs, and they are extremely effective at deterring cannibalistic conspecifics. Although the highly territorial nature of *P. cinereus* has been well studied (Jaeger *et al.*, 1982; Jaeger and Schwarz 1991; Jaeger and Peterson, 2002) aggressive behavior specific to nest guarding, perhaps due to its secretive nature, has been less well studied.

Ritualized territorial posturing, extended parental care, a fully terrestrial life cycle, and ease of maintenance in the laboratory make *P. cinereus* an ideal subject for the study of parental behavior. Brooding behavior is well documented in this species; however, few studies have specifically investigated environmental factors that contribute to the level of aggression demonstrated during nest guarding.

I investigated several criteria that parents might use to evaluate offspring value and level of threat. Females were very aggressive under multiple conditions, yet they increased aggression to an even higher level late in the season when guarding older eggs. Based on parental investment theory, I predicted that eggs closer to hatching should be guarded more fiercely than newly laid eggs, since they have higher reproductive value. Females behaved as predicted, with preferential guarding when egg value was high. The mechanism of clutch age recognition in *P. cinereus* is not yet understood. It is not clear whether these salamanders recognize developmental stage, or instead can

sense the amount of time passed or the time of year in order to gauge the level of defense required. The Forester *et al.* (2005) study suggests that at least one Plethodontid, *Desmognathus fuscus*, can recognize the developmental stage of eggs. However, this trait may vary between species due to differing selective pressures. Females did not guard 10 egg clutches more vigorously than 4 egg clutches as predicted; however, the high level of aggression achieved may be explained by the large reproductive value of both clutch sizes. The prediction that aggression should increase with maternal body size was also not supported. Both large and small females were aggressive, suggesting that females of all sizes are willing to incur the risk of injury defending their eggs. Nest guarding females also exhibited a slight trend towards increased aggression toward female rather than male intruders. Although this trend was not significant, it supports other studies in which non-brooding territorial females are more tolerant of male intruders than females. The high level of aggression exhibited under many conditions implies that parents probably do weigh costs against benefits and make decisions to defend offspring based upon circumstance, or upon egg or intruder characteristics.

In addition to examining aggressive behavior during nest guarding, I also examined an endocrine correlate of this behavior by measuring hormones in brooding females immediately following an intrusion to the nest. John Wingfield and his colleagues first showed a link between observed aggressive behavior and a physiological response in male birds. Androgen levels in monogamous parental male birds were elevated in response to a territorial intrusion (Wingfield

et al., 1990). The phenomenon was dubbed "The Challenge Hypothesis", and since the publication of this now classic paper, a plethora of research has examined the interaction between territorial aggression and steroid hormones in males and females of a wide variety of species. However, tests of the challenge hypothesis have yielded conflicting results, indicating the need for comparative data on understudied taxa. My evidence that the challenge hypothesis may apply to a nest guarding female amphibian represents the only study to directly demonstrate this phenomenon in an amphibian, and is only the third study to demonstrate an androgen response to a challenge in a female of any organism (Langmore, 2002; Desjardins, *et al.*, 2006); although a study of female marine iguanas demonstrated similar effects of estradiol (E_2) and progesterone (P_4), rather than testosterone (T) (Rubenstein and Wikelski, 2005).

Two possible conclusions may be drawn about the elevation of T in the brooding challenged female group: 1) aggression caused a rise in T or 2) the need for future aggression caused a rise in T. I favor the later explanation since some females in the challenged brooding group had elevated T but did not perform any aggressive behaviors; instead they simply curled around their eggs. In addition, some of the least aggressive females had the highest tissue T levels. Elevated testosterone may not cause behavioral changes, but may only increase the likelihood of an aggressive behavior in the presences of a certain stimuli. (Christiansen, 2001). The presence and magnitude of the testosterone surge in individual brooding residents may also be explained as a qualitative response to the behavior of the intruder in a given encounter.

I have demonstrated a novel technique for analysis of T, but not E_2, using autotomized tail tissue as an alternative to lethal or painful heart puncture. The male seasonal profile pattern I report from tail tissue is similar to patterns from blood plasma in many caudates, and from testicular tissue in *Salamandra salamandra*, which show T correlated to periods when males are competing for mates (Lecouteux *et al.,* 1995). The female profile is also consistent with *Salamandra salamandra* females, in which baseline T is elevated when females are gravid (Degani *et al.*, 1997). This technique may provide an alternative procedure for sampling hormones in endangered species with the ability to regenerate tissue.

A combination of my results along with comparison to existing studies in the literature underscore behavior differences among species and between individuals. Within a species, differences in reproductive status may explain variations in behavior. For instance, brooding females are more aggressive than both territorial gravid females and non-gravid females. Time of the year may also explain differences in behavior. For example non-brooding females may be more aggressive in spring than in summer, as the comparison of my results to Jaeger's (1982) study suggest. Also, spring gravid *P. cinereus* females have higher T than females at other times of the year. On the other hand, a brooding resident's behavioral response and/or tissue T titer may instead be best predicted by the location, behavior, or posture of intruders.

Many individual females were either consistently aggressive or consistently passive, while both aggressive behavior and T level varied widely between

individual brooding females. Some individuals within the brooding female group had elevated T but were not aggressive, while others were aggressive with low tissue T titres. These differences may indicate that additional variables such as past experience or age, shy/bold "personalities", or dominance status may be important in explaining the individual variations in behavior observed and variations in tissue T levels. Dominance hierarchies, which are prevalent in many taxa, have not been examined in *P. cinereus*.

Variation in temperament may also explain differences in aggression between individuals, although this has not been tested in an amphibian. The concept of temperament has been of growing interest to behavioral biologists. Temperament describes how an individual reacts in new or stressful situations. Wilson *et al.* (1994) proposed a shy-bold gradient that is classified by behavior; shy individuals retreat while bold individuals exhibit curiosity in novel situations. Temperament is predicted to be subject to phenotypic plasticity. Trade-offs between current and future reproduction may give rise to populations with polymorphic life history strategies; with bold individuals placing more emphasis on current reproduction and shy individuals placing more emphasis on future reproduction. The existence of both "personality" types in a population may be evolutionarily stable when both shy and bold individuals are selected for over evolutionary time as those best able to survive and reproduce.

Researchers are beginning to address the question of repeatable temperament traits in animals (Wolf *et al.*, 2007). In a study on bighorn ewes, individuals demonstrated highly repeatable "personality" traits, specifically

boldness and docility. Individuals captured on successive occasions were consistent in their measurements of temperament (Réale *et al.*, 2000). In ewes the personality trait termed "docility" was highly repeatable within individuals, and Réale suggests that this trait may be inherited; while boldness (which was less repeatable) may be subject to environmental influences such as past experience. In stickleback fish (*Gasterosteus aculeatus*) individuals that were bold when defending against predators were also more aggressive in social situations (Tulley and Huntingford, 1988). In contrast, studies on pumpkinseed sunfish (*Lepomis gibbosus*) show context dependent temperament measures (Coleman and Wilson, 1998). It remains to be seen whether temperament is context-specific or domain-general, whether a shy-bold continuum exists, and what the effects of environment are on temperament. Of late, evidence has been accumulating that personalities may be found in a wide variety of animals, and several studies using models have provided a plausible explanation for the evolution of personality types (Wolf *et al.* 2007). The question of repeatable temperament certainly merits further investigation in amphibians; and *P. cinereus* would make an excellent model for examining this phenomenon.

Both aggressive behavior during nest guarding and the physiological response in preparation for defense have been documented in a wide variety of taxa, and therefore are probably adaptive. Many studies have demonstrated that behavioral as well as endocrine responses by parents enhance the survivorship of offspring. Parents with elevated T may be physiologically prepared to fight and therefore better equipped to deter intruders. Both aggression during nest

guarding and the elevation of tissue T to support it are physiologically taxing, and parents must make trade-offs between devoting energy to their offspring and to their own energetic demands. My results suggest that individual brooding females are able to make decisions about nest guarding based on environmental cues such as clutch age; and that they have a mechanism (the elevation of T) which physiologically enables them to defend their progeny. Furthermore, variations among individual females' aggression scores and tissue T titres, as well as the consistency of individual brooding *P. cinereus* females to be either passive or aggressive, suggest that future research is warranted to examine the possibility of repeatable temperaments in an amphibian species.

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APPENDICES

APPENDIX A.

UNIVERSITY of NEW HAMPSHIRE

March 2, 2005

Scott, Michelle
Zoology
Rudman Hall
Durham, NH 03824

IACUC #: 050105
Approval Date: 01/28/2005
Review Level: B

Project: Hormones and Aggression in Brooding Red-Backed Salamanders

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category B on Page 4 of the Application for Review of Vertebrate Animal Use in Research or Instruction - *the study involves either no pain or potentially involves momentary, slight pain, discomfort or stress.*

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this study. Requests for extension must be filed prior to the expiration of the original approval.

Please Note:

1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. A Medical History Questionnaire accompanies this approval; please copy and distribute to all listed project staff who have not completed this form already. Completed questionnaires should be sent to Dr. Gladi Porsche, UNH Health Services.

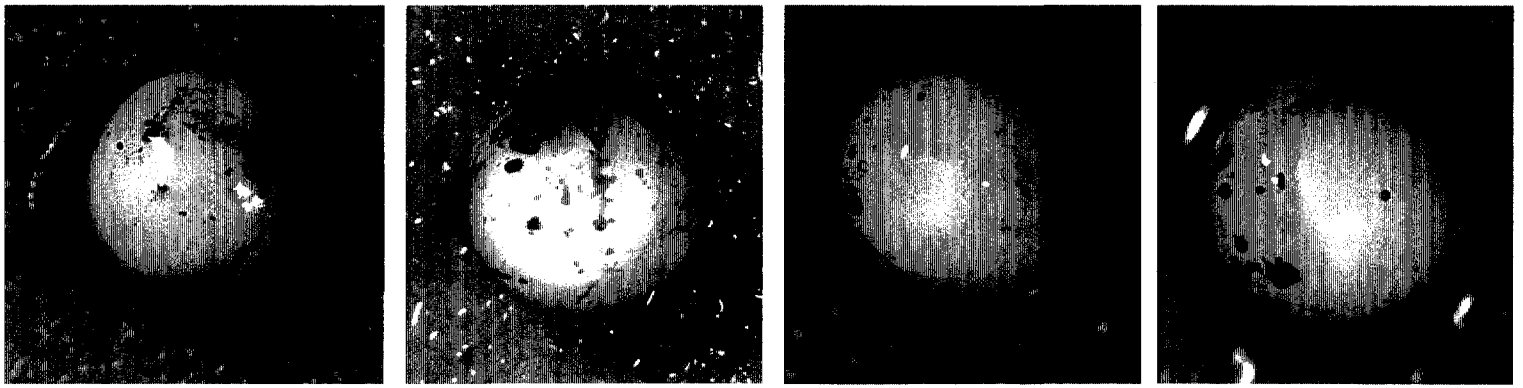
If you have any questions, please contact either Mae Sandler at 862-[illegible] or Julie Simpson at 862-2003.

For the IACUC,

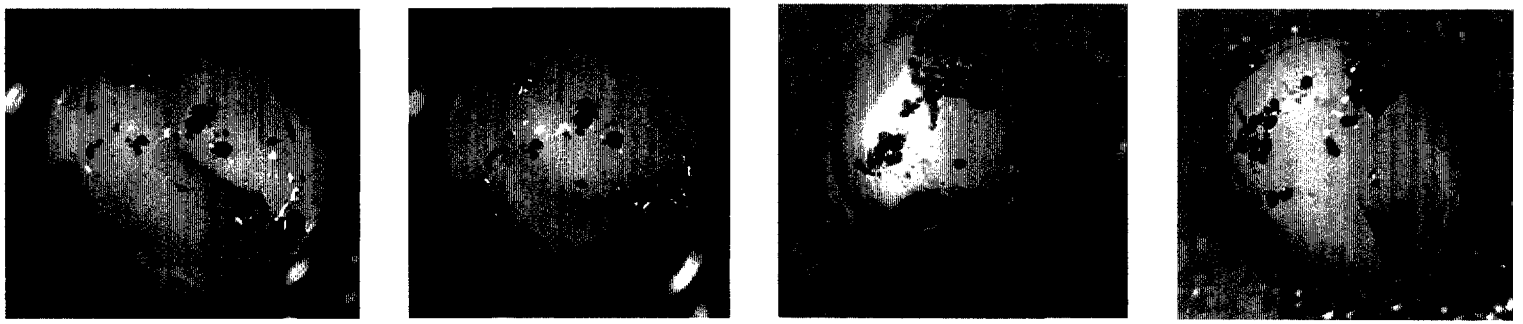
Robert G. Marr, Ph.D.
Chair

cc: File

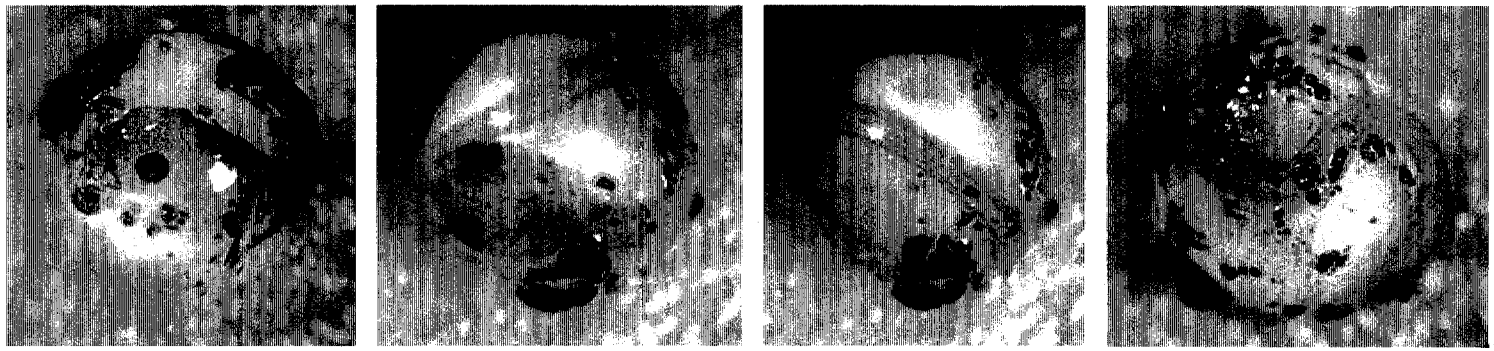
APPENDIX B. DEVELOPMENTAL PHOTOGRAPHS OF *P. CINEREUS* EGGS



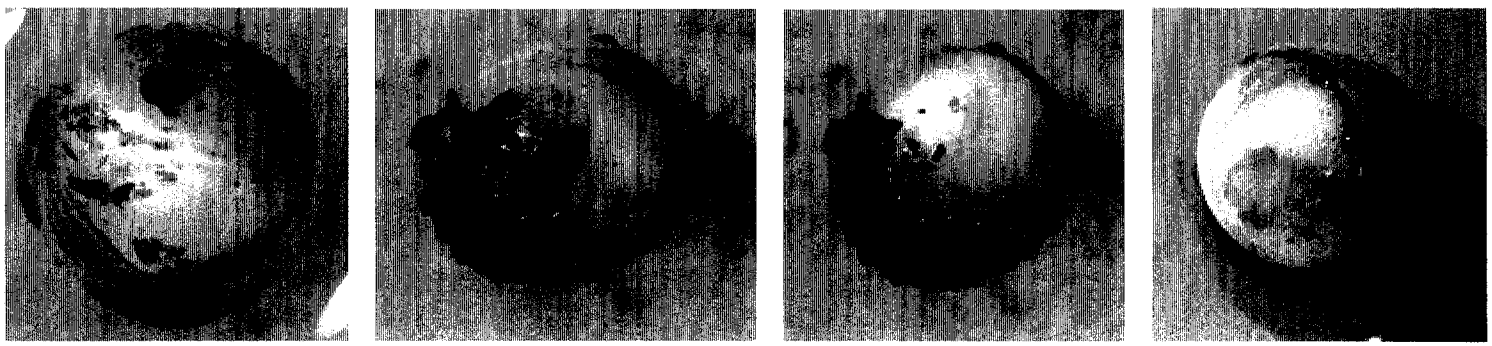
Week One- Gastrulation through early embryonic development occurs in the first week. In the first 2-3 days only yolk is visible, later pigment cells become visible, and some movement can be seen. All photos are taken with a digital camera through a dissecting microscope. An adhesive gel with bits of soil coats each egg.



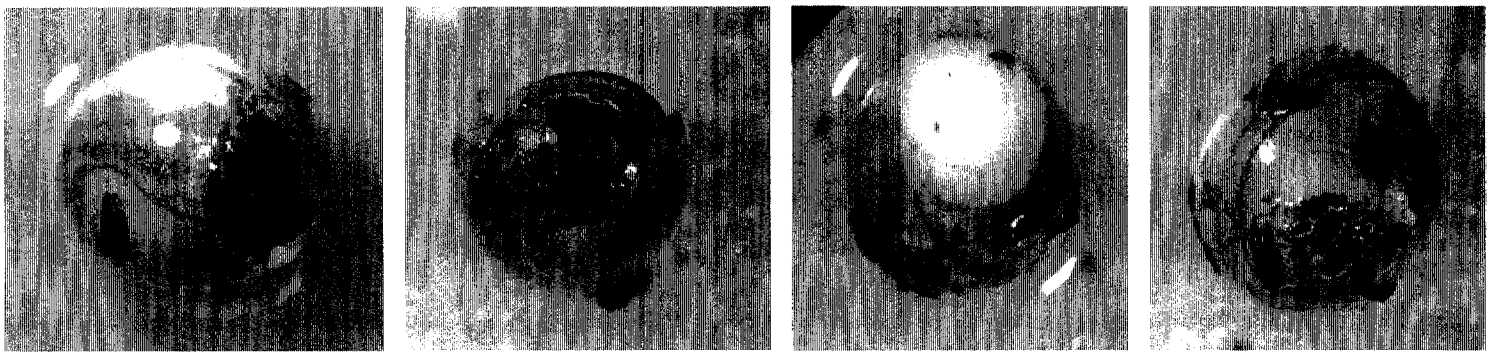
Week Two- Pigment becomes more prominent, face morphogenesis begins, and eyes are barely visible.



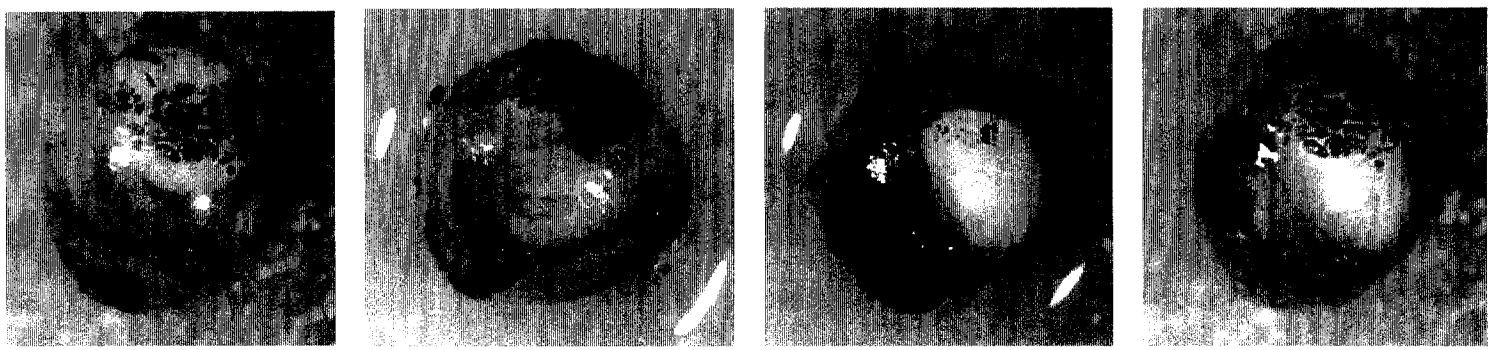
Week Three- Eyes become more advanced. By the end of the third week, blood vessels and gills are clearly visible, and red pigmentation appears along the back and tail.



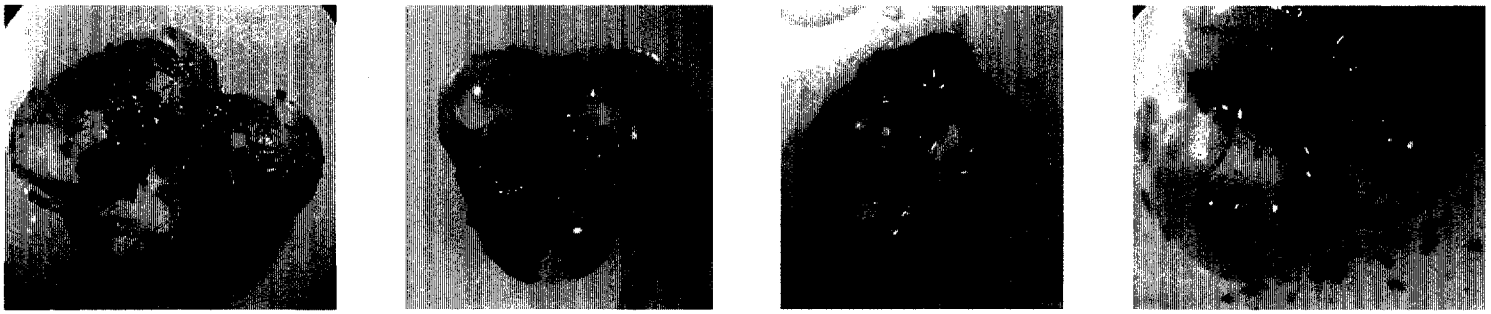
Week Four- Face and body become more developed. Larvae are still quite active at this stage.



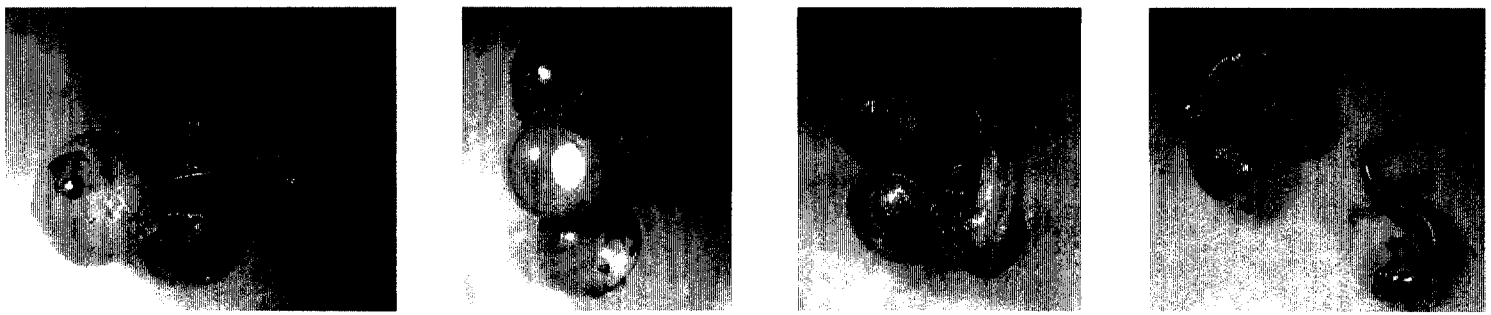
Week Five- Nares and hind limbs are visible, and digits are forming. The yolk sac is diminishing.



Week Six- Front and hind digits are fully formed. Movement becomes more restricted, and yolk sac is smaller yet.



Week Seven- Development continues and larvae resemble miniature adults at this stage. Movement is minimal.



Week Eight- Yolk sacs are almost completely dissolved. Hatching usually occurred in the laboratory around day 54-55.